INTERMITTENT HYPERCAPNIA INDUCES LONG-LASTING VENTILATORY PLASTICITY

TO ENHANCE CO_2 RESPONSIVENESS TO OVERCOME DYSFUNCTION

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A

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Abstract

The ability of the brain to detect (central CO_2 chemosensitivity) and respond to (central CO_2 chemoresponsiveness) changes in tissue CO_2/pH, is a homeostatic process essential for mammalian life. Dysfunction of the serotonin (5-HT) mechanisms compromises ventilatory CO_2 chemosensitivity/responsiveness and may enhance vulnerability to pathologies such as the Sudden Infant Death Syndrome (SIDS). The laboratory of Dr. Michael Harris has shown medullary raphé contributions to central chemosensitivity involving both 5-HT- and γ-aminobutyric acid (GABA)-mediated mechanisms. I tested the hypothesis that postnatal exposure to mild intermittent hypercapnia (IHc) induces respiratory plasticity, due in part to strengthening of bicuculline- and saclofen-sensitive mechanisms ($GABA_A$ and $GABA_B$ receptor antagonists respectively). Rats were exposed to IHc-pretreatment (8 cycles of 5 % CO_2) for 5 days beginning at postnatal day 12 (P12). I subsequently assessed CO_2 responsiveness using an *in situ* perfused brainstem preparation. Hypercapnic responses were determined with and without pharmacological manipulation. In addition, IHc-pretreatment effectiveness was tested for its ability to overcome dysfunction in the CO_2 responsiveness induced by a dietary tryptophan restriction. This dysfunctional CO_2 responsiveness has been suggested to arise from a chronic, partial 5-HT reduction imparted by the dietary restriction. Results show IHc-pretreatment induced plasticity sufficient for CO_2 responsiveness despite removal of otherwise critical ketanserin-sensitive mechanisms. CO_2 responsiveness following IHc-pretreatment was absent if ketanserin was combined with bicuculline and saclofen, indicating that the plasticity was dependent

upon bicuculline- and saclofen-sensitive mechanisms. IHc-induced plasticity was also capable of overcoming the ventilatory defects associated with maternal dietary restriction. Duration of IHc-induced plasticity was also investigated and found to last far into life (up to P65). Furthermore, I performed experiments to investigate if IHc-induced plasticity was more robust at a specific developmental period. No such critical period was identified as IHc-pretreatment induced robust respiratory plasticity when administered at all developmental periods tested (P12-16, P21-25 and P36-40). I propose that IHc-induced plasticity may be able to reduce the severity of reflex dysfunctions underlying pathologies such as SIDS.

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Acknowledgements

I have heard several horror stories about pursuing a graduate degree in the sciences. My experience has been exactly the opposite. I can honestly say that attending graduate school was never a plan of mine. However, if given the opportunity to do it all over again, I would pursue a graduate degree every time. I have many people to thank for my delightful experience in graduate school.

My advisor, Dr. Michael Harris, has played a huge part in my enjoyment of graduate school. Dr. Harris allowed me as much independence as I wanted, but was always immediately available for advice and to answer questions when I needed him. His obvious passion for science is truly inspiring, and I give him much credit for my desire to continue in research. Looking back I have had several absolutely terrible ideas for avenues in which to take my research. Dr. Harris always listened respectfully and considered each of my ideas, no matter how bad they were. This respect for students' curiosity has garnered a great learning atmosphere in which I was able to think freely without fear of being wrong or proposing a far-fetched theory to investigate. Outside of the lab, Dr. Harris has been nothing but 100 % supportive of my other academic pursuits. An example of this unwavering support was when he allowed me to spend four months at the National Institutes of Health (NIH) to pursue additional research interests. Furthermore, not only did Dr. Harris allow me to go to the NIH, but from what I'm told he wrote a glowing letter of recommendation on my behalf. Having Dr. Harris as my advisor undoubtedly has made my graduate experience the enjoyable endeavor that it has been.

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Just as I have heard horror stories about graduate school, I have also heard unsettling stories about graduate committees. Again, my experience has been exactly the opposite. I feel extremely fortunate to have such a supportive committee that has helped me through each step in the graduate process and who have taught me more that I could ever describe.

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Dedication

I would like to dedicate the work of this dissertation to all the fallen Sprague-Dawleys who selflessly sacrificed themselves in an effort to increase the understanding of the mammalian respiratory network and improve the health and wellbeing of all. Without the sacrifices of these brave, furry heroes, the findings described herein would have been impossible.

Chapter 1

Introduction

1.1 Overview of central control of breathing

Breathing is an essential homeostatic process that allows precise regulation of oxygen (O_2) and carbon dioxide (CO_2) concentrations in blood and tissues. Within the medulla of the mammalian brainstem exist vital circuits that control respiratory movements and maintain homeostasis of the brain and body. The seemingly simple, automatic process of breathing is in fact the result of complex neural mechanisms.

Respiration is thought to arise from two independent oscillators located within the ventrolateral medulla: the pre-Bötzinger complex (pre-BötC) that is widely considered to be the inspiratory kernel (Smith et al., 1991), and the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) that plays a major role in active expiration (Fig. 1.1). The subset of pre-BötC neurons thought to be responsible for inspiration possess intrinsic bursting properties with two main underlying mechanisms. The first is dependent upon sub-threshold activation of persistent Na^+ current (I_{NaP}) in neurons with sufficiently low leakage-like K^+ current and is voltage sensitive (Koizumi and Smith, 2008; Del Negro et al., 2002; Butera et al., 1999). The second bursting mechanism is less voltage sensitive and depends on Ca^{2+}-activated nonspecific cationic current (I_{CAN}), whose activation mecha-nism in the absence of synaptic input depends on voltage-gated Ca^{2+} channels (Smith et

al., 2007). This pre-BötC neuronal population drives inspiratory activity through projections to various premotor populations, such as the rostral ventral respiratory group (rVRG) and the parahypoglossal (pXII) group, that in turn project both to inspiratory muscles that pump air, mainly the diaphragm and external intercostals, and to inspiratory muscles that modulate airflow resistance, such as laryngeal and tongue muscles (Feldman et al., 2012).

At rest, expiration is generated passively due to the forces generated by the elastic recoil of the inspiratory muscles, lung and rib cage. However, during situations such as exercise, encountering an elevated CO_2 stimulus or a powerful emotional response, non-chemosensitive neuronal populations in the RTN/pFRG region are activated and function to generate active expiration. The RTN/pFRG drives active expiratory activity through projections to various premotor populations, such as the caudal ventral respiratory group (cVRG) and the pXII group, which in turn project both to expiratory pump muscles, abdominals and internal intercostals, and to expiratory muscles that modulate airflow resistance, such as laryngeal and tongue muscles (Feldman et al., 2012).

The RTN/pFRG and preBötC, together, act as coupled oscillators, and comprise the central rhythm generator (CRG), which produces respiratory rhythm. In addition, several neuronal populations interact with the pFRG and preBötC to modulate respiratory output (Figs. 1.1 and 1.2). One such modulatory input to the respiratory CRG is the central response to CO_2, known as central chemoreception.

Cells that possess central CO_2 chemoreception abilities are acid-sensing and stimulated by CO_2/pH in the blood, cerebral spinal fluid or tissue. When blood CO_2 levels increase, CO_2 diffuses across the blood brain barrier capillaries to equilibrate with cerebral spinal fluid or tissue and the resulting acidosis stimulates chemoreceptors. The result of such stimulation, referred to as the hypercapnic ventilatory response (HCVR), varies depending on neuron type, however, the eventual output triggers an increase in respiratory rate and amplitude. The HCVR is a critical reflex in mammals and is essential for the maintenance of homeostasis. Several regions in the brainstem have been identified at potential sites of CO_2 chemoreception.

In the medullary raphé nuclei of the brainstem, a subset of serotonin (5-hydroxytryptamine or 5-HT) -synthesizing neurons act as central respiratory chemoreceptors (CRCs). The medullary raphé provides tonic modulatory input to the respiratory network through 5-HT neuronal activation (Corcoran et al., 2009). However, depending on the animal model, experimental preparation and developmental period, 5-HT receptor activation is capable of eliciting either excitatory or inhibitory effects on respiratory control.

1.2 Introduction to central CO_2 chemosensitivity

CO_2-sensitive central chemoreceptors provide critical sensory information, which affects synaptic drive necessary for rhythm generation and modulates respiratory pattern to protect the brain from excessive changes in CO_2 and pH.

Breathing depends on input from chemoreceptors, which provide the essential information about O_2, CO_2 and pH (Nattie, 1999). The majority of O_2-sensitive chemoreceptors are located outside the brain in the carotid bodies. CO_2/pH-sensitive chemoreceptors are found in the carotid bodies, however, major sites are also within the brain and are referred to as CRCs. CO_2/pH signals are related to the acid-base status of the blood and brain and even small changes in CO_2/pH can affect breathing.

To be considered a chemoreceptor, a neuron must not only be chemosensitive, but it must also be intrinsically chemosensitive, or responsive to pH/CO_2 change without input from another chemoreceptor. In addition, this network-independent chemosensitivity must also occur over a physiologically relevant range of CO_2 concentrations and pH, and ultimately modulate ventilatory output to rectify the pH homeostatic challenge.

Central CO_2 chemosensitivity is considered a complex system function that involves a limited but varied group of neuron types, brain stem sites and multiple neurotransmitter mechanisms (Fig. 2; Feldman et al., 2003; Nattie, 2009; Nattie and Li, 2009; Putnam et al., 2004). Although much controversy surrounds the critical center for central CO_2 chemosensitivity, research has yet to definitively identify such a region. The wide range of findings concerning central CO_2 chemosensitivity may be due to the numerous ways in which it is investigated. Differences in experimental preparations (*in vivo, in situ* or *in vitro*) can have significant effects on conclusions drawn about central chemosensitivity, and with each preparation comes its own limitations. Because CRCs are likely in-

volved in complex networks, and the necessity of isolating populations of intrinsic chemoreceptors, experimental isolation *in vivo* can be very challenging. In an attempt to overcome such limitations, various experimental preparations are commonly utilized when drawing conclusions about central respiratory chemoreception.

Discrete sites that are very sensitive to small changes in pH function in concert to establish system CO_2 chemosensitivity. Many cell types and molecular mechanisms are involved in the process of CO_2 chemoreception. Examples of potential pH-sensitive proteins present at many chemoreceptor locations include (i) low resistance gap junctions (Solomon et al., 2001); (ii) TWIK-related acid-sensing K^+ (TASK) channels, such as transient receptor potential channel canonical subfamily 4 (TRPC4) and 5 (TRPC5; Cui et al., 2011; Semtner et al., 2007); (iii) inward rectifier K^+ channels (Jiang et al., 2001); and (iv) pH-sensitive membrane ion transport proteins, such as the Na^+/H^+ exchange protein subtype 3 (NHE3), which may modulate CO_2 chemosensitivity by altering the degree and timing of intracellular pH changes (Putnam, 2001). In addition, it is possible that more than one pH-sensing function may operate simultaneously. Neurotransmitters and neuromodulators that are involved in the central response to CO_2 include orexin, adenosine triphosphate (ATP), thyrotropin releasing hormone (TRH), noradrenalin, substance P (SP), 5-HT, γ-aminobutyric acid (GABA) and glutamate, in gaseous neurotransmitters (e.g., nitric oxide). The receptors for these neurochemicals are located postsynaptically on chemoreceptors in the brainstem (Hodges and Richerson, 2010b; Huckstepp and Dale, 2011; Putnam, 2001).

1.3 Raphé 5-HT and GABA neurons as CO_2 chemosensors

In addition to being sensitive to small changes in pH, medullary 5-HT neurons have a convenient relationship with blood vessels that affords them a specialized role as arterial CO_2 sensors (Bradley et al., 2002). Most of the blood flow to the medulla travels through the midline raphé in branches of the basilar artery (Fig. 1.3). Partial pressure of CO_2 (P_{CO_2}) within the raphé closely reflects the P_{CO_2} of blood coming out of the lungs as there is a high density of large arteries in this region, and they are located proximal to the arterial tree, which is one of the first branches after leaving the heart. 5-HT neuronal dendrites wrap around the walls of the large midline arteries which allows them to take full advantage of the vascularity in this location. Amazingly, in some cases the dendrites come within one half of a micrometer from the vessel lumen. The unique relationship between raphé neurons and large arteries is thought to provide a functional advantage allowing the brain to monitor the P_{CO_2} of blood after it has come out of the lungs, and before being influenced by brain metabolism (Bradley et al., 2002). This amazingly convenient design is an excellent way to monitor the effectiveness of lung ventilation and prevent sudden increases in brain P_{CO_2}.

A subset of medullary raphé neurons is highly sensitive to changes in CO_2 and pH. In rat brain slices, some medullary raphé neurons are strongly stimulated by an increase in P_{CO_2} from 33 mmHg to 60 mmHg, and are inhibited by a decrease in P_{CO_2} from 33 mmHg to 23 mmHg (Richerson, 1995). Despite this finding, most medullary raphé neurons

do not respond to changes in P_{CO_2} within these ranges and most medullary raphé neurons are not serotonergic. In order to eliminate the effects of synaptic input, increase recording stability and have enhanced experimental control, chemosensitive medullary raphé neurons can be isolated in tissue culture. Consistent with findings in brain slices, a subset of raphé neurons in culture is stimulated by increased CO_2. However, a different subset of neurons is inhibited by the same CO_2 stimulus. In addition, the morphologies of these two types of chemosensitive neurons are different, indicating that they are phenotypically distinct (Wang et al., 1998). This possibility was further solidified by the finding that all CO_2-stimulated neurons from the medullary raphé, but none of the CO_2-inhibited neurons, are serotonergic (Wang et al., 2001). In addition to acidosis-stimulated 5-HT neurons found in the raphé, *in vitro* analyses have identified the acidosis-inhibited population of neurons as being GABAergic (Iceman et al., 2010; Wang et al., 1998). Despite much evidence indicating that the raphé nuclei in the medulla possess network-independent intrinsic chemosensitivity *in vitro* (Corcoran et al., 2009; Hodges and Richerson, 2010a), the role of raphé neurons in chemosensitivity *in vivo* is a topic of ardent debate.

Despite much disagreement concerning what types of neurons are the critical chemosensors, much evidence suggests that 5-HT neurons play a role in chemosensitivity (Richerson, 2004). Because 5-HT neurons are intrinsically chemosensitive *in vitro*, are stimulated by hypercapnia *in vivo* and this response is attenuated when 5-HT neurons are disrupted, 5-HT neurons are thought to function as chemoreceptors. Further strengthening the case for 5-HT neurons as chemosensors is that when the 5-HT_2 receptor antagonist

ketanserin is applied *in situ*, the response to hypercapnia is abolished (Corcoran et al., 2013). In addition *in vivo* inhalation of CO_2 increases c-Fos staining in the medullary raphé of cats (Larnicol et al., 1994). This finding was later confirmed and extended when it was shown that CO_2-activated neurons in the medulla of rats are immunoreactive for 5-HT and tryptophan hydroxylase (Haxhiu et al., 2001; Johnson et al., 2003). Furthermore, microinjection of azetazolamide, a carbonic anhydrase inhibitor that acts to induce tissue acidosis, causes an increase in breathing when focally injected in the medullary raphé of rats (Bernard et al., 1996). Later experiments demonstrated that ventilation in rats is also stimulated by microdialysis of a high CO_2 solution in the medullary raphé (Nattie and Li, 2001). Further demonstrating their role as chemoreceptors, lesioning medullary 5-HT neurons leads to blunting of the response to hypercapnia (Richerson, 2004). Additionally, rats exhibit a depressed hypercapnic response when serotonergic neurons in the medullary raphé are inhibited using 8-OH-DPAT, a 5-HT_{1A} receptor antagonist (Messier et al., 2004).

In addition, several genetic methods have been used to investigate 5-HT and its role in the response to CO_2. Pet-1 knockout mice, which lack ≈ 70 % of 5-HT neurons, display reduced CO_2 sensitivity in males but not females (Hodges et al., 2011). Furthermore, neonatal mice with genetic deletion of *Lmx1b* in neurons expressing *Pet-1* (*Lmx1b*$^{f/f/p}$), which have a selective and severe deficit (> 99 %) in 5-HT neurons, display a ≈ 50 % reduction in the hypercapnic ventilatory response (Hodges and Richerson, 2008;

Hodges et al., 2008). These examples of various levels of 5-HT neuron reductions further demonstrate the important role that 5-HT plays in the response to hypercapnia.

Despite the impressive body of research on 5-HT raphé as chemosensors, this hypothesis does not imply that 5-HT or any raphé neurons are the exclusive sensory transducers of hypercapnia in the brain. Instead, it is more likely that they are one component of what is probably a network of chemosensitive brain sites (Richerson, 2004).

1.4 Push-pull model of raphé chemosensitivity

The laboratory of Dr. Michael Harris has developed a model describing raphé contributions to central CO_2 chemosensitivity wherein both 5-HT and GABA mechanisms modulate ventilation to maintain tissue CO_2/pH through synaptic activation and disinhibition, respectively (Fig. 1.4; Corcoran et al., 2008; Richerson, 1995). We have demonstrated that these cell types retain CO_2 chemosensitivity in the intact and unanesthetized brainstem (Corcoran et al., 2008, 2013; Iceman et al., 2013).

Hypercapnia stimulates raphé 5-HT neurons and activates CRGs and motor neuron pools (MNP), which causes an increase in ventilation. Under normocapnic conditions (normal levels of CO_2), GABA raphé neurons provide tonic inhibitory input to raphé 5-HT neurons, MNPs and CRGs. However, when exposed to hypercapnia, GABA neurons are inhibited. This inhibition of GABA neurons disinhibits the 5-HT cells, MNPs and CRGs, leading to an eventual stimulation of ventilation. When exposed to low levels of CO_2 (hypocapnia), 5-HT neuron drive is inhibited and GABA neurons are excited. This excitation of

GABA neurons has an inhibitory influence on 5-HT neurons, MNPs and CRGs. The combined inhibition of 5-HT neurons and excitation of GABA neurons brought on by hypocapnic conditions leads to a depression of ventilation.

1.5 Evidence for other central CO_2 chemoreceptors

There is strong evidence that there are additional central CO_2 chemoreceptors located in other brainstem nuclei besides the raphé, including the RTN, locus coeruleus (LC), nucleus tractus solitarious (NTS), lateral hypothalamus and cerebellum. Central CO_2 chemosensitivity is considered a complex system function that involves several brain stem sites (Fig. 1.1; Feldman et al., 2003; Nattie, 2009; Nattie and Li, 2009; Putnam et al., 2004). The RTN is well-established as being important in respiratory control and central CO_2 chemoreception (Feldman et al., 2003; Guyenet et al., 2005; Li and Nattie, 1997). RTN neurons have a large response to CO_2 inhalation *in vivo* (Mulkey et al., 2004), however, because it is not known how much of their response is intrinsic, it remains unclear how important these neurons are as chemoreceptors (Corcoran et al., 2009). Given that several neuronal groups function in the response to hypercapnia, if one chemosensory region is rendered dysfunctional as a result of injury or disease, other regions may be able to compensate to mediate an appropriate response.

1.6 Breathing disorders associated with 5-HT dysfunction

Serotonin plays an important role as a signaling molecule, acting as a neurotransmitter in all species of the animal kingdom (Azmitia, 2007; Berger et al., 2009). In the mammalian central nervous system (CNS), the relatively small number of serotonergic neurons are located near and along the midline of the brainstem. Despite being a small population, these few neurons have projections to target regions throughout the entire neuraxis. The effects of 5-HT, and co-released neuropeptides such as SP and TRH, depend upon their combination of receptors, the second messenger systems and the developmental stage in which they are expressed. With diverse projections and numerous pre- and post-synaptic receptor subtypes, it is not surprising that the 5-HT system has been proposed to contribute to numerous brain functions and pathologies. These include, but are not limited to, neurogenesis, synaptic plasticity, brain homeostasis, sleep and circadian rhythms, appetite, pain, thermoregulation, micturition, addiction, migraine, depression, fear and anxiety, aggression and rage, learning and memory, obsessive compulsive disorder, schizophrenia, Prader-Willi syndrome, autism, and breathing disorders such as Sudden Infant Death Syndrome (SIDS), sleep apnea and Congenital Central Hypoventilation Syndrome (CCHS).

Research on SIDS victims has uncovered an abnormality in the medullary serotonergic system (Kinney et al., 2001), including the medullary raphé (Panigrahy et al., 2000). This evidence supports the hypothesis that SIDS victims are not normal prior to death, despite appearing to be in good health (Filiano and Kinney, 1994). In one study of

84 cases at The Boston Children's Hospital over a 10-year period (1985–1995), mean 5-HT binding was analyzed in 52 SIDS cases (cause of death undetermined), 15 acute controls (cause of death established at autopsy) and 17 chronic controls (deaths attributed to known oxygenation disorders) (Panigrahy et al., 2000). Nineteen brainstem nuclei, including the medullary raphé, were analyzed for [3H]-lysergic acid diethylamide ([3H]-LSD) binding, a 5-HT receptor agonist binding to $5HT_{1A-D}$ and $5HT_2$ receptors. Results indicated reduced 5-HT receptor binding in the raphé compared to either the acute controls or chronic controls. The 50–60 % decrease in mean 5-HT receptor binding found suggests an abnormality in the medullary raphé of SIDS victims (Panigrahy et al., 2000). Because the raphé is involved in the modulation of a variety of homeostatic processes, including respiratory control, abnormalities in this brainstem region could prevent a vulnerable infant from responding to a life-threatening challenge, such as hypercapnia, during sleep.

A "Triple Risk Model" has been suggested as an appropriate method in which to describe the interaction of multiple factors in the pathogenesis of SIDS (Filiano and Kinney, 1994)**.** According to this model, SIDS results when three factors simultaneously influence an infant: (a) an underlying vulnerability in the infant, (b) a critical developmental period and (c) an exogenous stressor, e.g., prone sleep position (Fig. 1.5). In the context of the Triple Risk Model, the risk factors for SIDS can be divided into extrinsic and intrinsic categories. Extrinsic risk factors are acquired physical stressors related to the circumstances of death, e.g., prone sleep position. Such exogenous stressors are postulated to induce asphyxia, hypercapnia and hypoxia. Intrinsic risk factors, however, are associated

with the underlying vulnerability/abnormality in the infant and increase the likelihood of SIDS by exacerbating this abnormality. The intrinsic risk factors include prematurity, male gender, African American race, poverty, adverse prenatal factors such as maternal smoking or alcohol use during pregnancy and genetic polymorphisms (Iyasu et al., 2002; Moon et al., 2007). The critical period for SIDS (the first six months of life) coincides with dramatic and rapid changes in the respiratory control system (Filiano and Kinney, 1994). According to the Triple Risk Model, only infants with an underlying brainstem abnormality die of SIDS. Because not all infants have an underlying brainstem abnormality, this explains why not all infants who are put to sleep in the prone position or who bed share die of SIDS. The model also explains why SIDS rates are reduced by the change to supine sleep position: The exogenous stressor is removed, which allows the vulnerable infant to pass through the critical period unharmed.

Sleep-disordered breathing, sleep apnea, is the periodic cessation of breathing during sleep. Approximately 5 % of middle-aged males and 2 % of children have sleep-disordered breathing (Bixler et al., 2001; Gaultier, 2001). Sleep apnea is associated with age-related dysfunction in the 5-HT system (Kubin et al., 1998). These defects could underlie deficits in upper airway tone during sleep, leading to apneas.

Central chemoreception is absent or reduced in children with CCHS (Spengler et al., 2001). These children function relatively normally during wakefulness but require ventilatory support during sleep to avoid very high CO_2 and low O_2 levels due to inadequate ventilation. Mutation of the human *Phox2b* gene has been identified in patients suffering

from CCHS. An animal model suggests that CCHS is due to a *Phox2b* gene mutation that deletes neurons expressing this transcription factor in the RTN/pFRG (Thoby-Brisson et al., 2009). This reduces phrenic nerve activity, alters respiratory frequency and attenuates system responses to CO_2.

Respiratory insufficiency is the major cause of morbidity and mortality in spinally injured patients (Ramer et al., 2000). Serotonin-dependent plasticity may play a critical role in spontaneous and induced functional recovery following spinal cord injury (Golder et al., 2001). Additional respiratory disorders have implicated 5-HT in their pathophysiological mechanism, including panic anxiety hyperventilation disorders (Klein, 1996) and ventilatory instability in Rett Syndrome (Dunn and MacLeod, 2001).

1.7 The role of nutrition in breathing disorders

During development, the respiratory control system is known to be sensitive to a variety of environmental perturbations, including psychological stress (maternal separation), chronic changes in respiratory gases (hypoxia, hyperoxia, hypercapnia), numerous drugs such as caffeine, nicotine, alcohol, (Bavis and Mitchell, 2008) and nutrition, especially maternal nutrition (Penatti et al., 2011).

Protein malnutrition affects a large population of infants, especially in developing countries. Several ontogenic steps of brain development such as neuronal proliferation and migration, brain growth spurt and myelination are altered by protein malnutrition in animal models (Gressens et al., 1997). Penatti et al. (2011) provided female rats with

chow containing 45 % less of the essential amino acid tryptophan, the essential precursor for 5-HT, than normal chow. Consequently, when these rats were bred, their offspring experienced low tryptophan availability throughout gestation and postnatal development. The tryptophan-restricted pups exhibited significantly reduced brainstem levels of 5-HT as expected, but they also exhibited a partially blunted response to hypercapnia. This observation confirms that a mother's nutritional status can influence her offspring's respiratory control system, at least during the neonatal period. These findings also indicate that malnutrition may be a mechanistic link between poverty and increased SIDS risk (Bavis, 2011). Part of my research repeated the dietary restriction utilized by Penatti et al. (2011) to induce a partial chronic 5-HT reduction. This was done to test the efficacy of a treatment aimed to reverse or combat the ventilatory dysfunction imparted by the 5-HT reduction.

1.8 Plasticity within central respiratory chemosensitivity

Plasticity is a unique and fundamental characteristic of neural systems enabling animals to adapt to changes in environmental conditions and behavior. Plasticity is a persistent change in the morphology and/or function of the neural control system based on prior experience. Relevant experiences include neural activity, hypoxia, hypercapnia, injury, disease or aging. A considerable amount of research in recent years has revealed that the neural mechanisms controlling respiration are capable of exhibiting remarkable plasticity (Mitchell and Johnson, 2003).

Central chemoreception is likely best described as a complex system function that involves a limited but varied group of neuron types, brainstem sites, and multiple neurotransmitter mechanisms (Feldman et al., 2003; Mitchell et al., 1990; Nattie, 2009; Nattie and Li, 2009; Putnam et al., 2004). Organization of chemosensitivity as a system function affords the potential for considerable plasticity. Not only could a specific mechanism play a differential role in overall system sensitivity under specific conditions, the involvement of multiple mechanisms may allow homeostatic regulation despite partial dysfunction, injury or disease (Feldman et al., 2003).

Various protocols using different chemoreceptor stimuli (hypoxia and hypercapnia), durations, intensities and patterns have been shown evidence of inducing distinct forms of plasticity in respiratory control. These resulting forms of plasticity differed in their effect (facilitation or depression) on different ventilatory parameters. In addition, the duration over which plasticity is evoked has been quite variable, ranging from seconds to years (Moore, 2000; Wagner and Eldridge, 1991). With such a wide range of plasticity being the result of the aforementioned protocols, it appears that the specific stimulus paradigm seems to be of great importance.

Hypoxia-induced respiratory plasticity is widely considered to be the most thoroughly studied and best understood form of respiratory plasticity. Depending on the specific hypoxia protocol, various forms of plasticity may be induced. In anesthetized rats, after a single hypoxic episode, a short-term depression of phrenic motor output (post-

hypoxia frequency decline) is observed (Coles and Dick, 1996). However, when intermittent hypoxia is administered, various unique forms of plasticity are induced. Elevated respiratory activity during normoxic exposures between successive hypoxic episodes is often observed, reflecting the development of long-term facilitation (LTF; Powell et al., 1998). Persistent elevation of respiratory motor output, lasting minutes to hours, is the result of 3-10 hypoxic episodes with each episode duration varying depending on experimental protocol (Mitchell et al., 2001). Furthermore, LTF is elicited by intermittent, but not continuous, hypoxia (Baker and Mitchell, 2000). If intermittent hypoxia persists, different mechanisms of plasticity are evoked. For example, chronic intermittent hypoxia augments the short-term hypoxic ventilatory response, eliminates post-hypoxia frequency decline and enhances LTF in rats (Ling et al., 2001). Just as different hypoxic stimulus protocols vary in their capacity to evoke respiratory plasticity, hypercapnic stimulus protocols also induce respiratory plasticity to varying degrees.

Although receiving less attention, and relatively poorly understood, hypercapnia is also capable of eliciting various forms of respiratory plasticity. In contrast to intermittent hypoxia, intermittent hypercapnia (≈ 10 % inspired CO_2) elicits long-term depression (LTD), a long-lasting decrease in the frequency and amplitude of respiratory motor output (Bach and Mitchell, 1998). However, LTD is not evoked by less severe levels of hypercapnia (≈ 5 %). In contrast to the previous report by Bach and Mitchell (1998), Baker et al. (2001) found that intermittent hypercapnia did not elicit significant LTD of phrenic ampli-

tude, but significant LTD of burst frequency was resolvable. In contrast to episodic hypercapnia, Baker et al. (2001) found that continuous hypercapnia did elicit prolonged LTD. Steggerda et al. (2009) examined the effects of daily exposure to intermittent hypercapnia on the ventilatory response to subsequent hypercapnic and hypoxic exposure in neonatal rat pups. In response to a subsequent hypercapnic challenge, there was no significant difference in the ventilatory response between control and intermittent hypercapnia-exposed groups. In contrast, intermittent hypercapnia-exposed rat pups exhibited an enhanced ventilatory response to hypoxic challenge with an increase in minute diaphragmatic electromyogram (EMG; Steggerda et al., 2009). In addition, rat pups that were exposed to perinatal hypercapnia exhibited only a transient reduction in the hypercapnic ventilatory response (Bavis et al., 2006). Collectively, these data suggest that the duration, intensity and pattern of chemosensory stimuli protocol utilized are of great importance when investigating the respiratory plasticity evoked by both hypoxia and hypercapnia. One aspect of my studies was dedicated to the investigation of respiratory plasticity induced after administration of an intermittent hypercapnia protocol during a specific developmental period in rats.

1.9 Potential mechanisms of respiratory plasticity

Although detailed cellular or synaptic mechanisms surrounding respiratory plasticity are yet to be revealed, several mechanisms have been suggested. It may be that respiratory

plasticity is induced by previous activity at a synapse, known as “activity-dependent synaptic plasticity.” While high-frequency activity is capable of enhancing synaptic transmission for hours to days (LTP; Bach and Mitchell, 1996; Malenka and Nicoll, 1999), low-frequency activity may decrease synaptic strength for minutes to hours (LTD; Fig. 1.6; Kemp and Bashir, 2001). In another form of plasticity, neurochemicals such as 5-HT, norepinephrine or trophic factors such as brain-derived neurotrophic factor (BDNF) may initiate or regulate synaptic plasticity, termed “neuromodulator-induced synaptic plasticity” (Fig. 1.6; Kinkead et al., 2001; Kovalchuck et al., 2002). In addition, it may be that existing, but ineffective, synaptic pathways or “silent synapses” are enhanced by experimental manipulations or injury resulting in respiratory plasticity (Fig. 1.6; Poncer and Malinow, 2001). Furthermore, respiratory plasticity may be induced by altered concentrations of neuromodulators near their targets, which may cause changes in the activity of neuromodulatory neurons, the number and size of neuromodulatory terminals, reuptake of neuromodulator once released, or synthesis and degradation of neuromodulators. In addition, plasticity also depends on the density and type of receptors on pre- and postsynaptic targets, as well as their intracellular signaling mechanisms (Fuller et al., 2002). Also, plasticity may be induced by changes in neuronal properties such changes in soma and dendrite size/shape (Fig. 1.7), alterations in membrane potential, input resistance, capacitance and action potential threshold (Cameron and Nunez-Abades, 2000; Luscher and Frerking, 2001). Further yet, plasticity may be due to the formation of new synaptic connections between existing neurons (Fig. 1.7; Cameron and Nunez-Abades, 2000).

1.10 Critical periods in the development of central CO_2 chemosensitivity

The development of the respiratory control system is complex and consists of dynamic interactions that begin early in gestation and, in mammals, does not achieve maturity until weeks or months after birth. In addition to its elaborate interactions and connections, the respiratory control system is capable of exhibiting plasticity induced by experience or training during "critical" periods of development. That is, the same experience occurring outside of the critical period has little or no lasting effect, indicating that the plasticity depends on time windows during ontogeny when development can be altered in response to the external environment. Thus, developmental exposure to numerous experiences such as episodic or chronic hypoxia, hyperoxia, hypocapnia or hypercapnia, or drug or toxin exposures may cause disruption of neural respiratory control maturation.

The development of central CO_2 chemosensitivity also appears to develop with a shift in the degree of responsiveness to CO_2. Wang and Richerson (1999) observed in medullary slices that the percentage of neurons stimulated by hypercapnia was significantly greater in slices from rats older than P12 compared to younger rats. In addition, these findings paralleled to those found in medullary raphé neurons in tissue culture (Wang and Richerson, 1999). These findings may reflect the suggested critical period described by the transient imbalance between excitatory and inhibitory neurotransmission at P12 (Liu and Wong-Riley, 2002). Given these findings, I chose to administer the intermittent hypercapnia protocol for five consecutive days beginning at P12.

1.11 The role of peripheral chemosensors

Both central (brainstem) and peripheral (carotid bodies) respiratory chemoreceptors play an important role within the respiratory control system to ensure that blood gases do not rise or fall with potentially negative, or even fatal consequences. Peripheral chemoreceptors respond to changes in blood gases in 3-5 seconds, which is approximately an order of magnitude faster than central chemoreceptors (Carroll et al., 1991). Carotid bodies are known to be the primary oxygen sensors but also play an important role in sustaining eupneic ventilation under normoxic conditions (Forster et al., 2008). In addition, carotid bodies are reported to respond vigorously to changes in P_{CO_2} (Cunningham, 1987).

Carotid body afferents mainly terminate on NTS neurons that have reciprocal connections not only with the RTN (a known region of CO_2 chemoreception) but also with many other neurons within the respiratory network, some of which are also chemosensitive (Rosin et al., 2006; Takakura et al., 2006). Research concerning the role of peripheral chemoreceptors is much debated. In general it is thought that peripheral chemoreceptors have three potential interaction modes with central chemoreceptors: hypoadditive, additive and hyperadditive (Teppema and Smith, 2013).

1.12 Comparison of *in vitro*, *in situ* and *in vivo* CO_2 chemosensitivity findings

Investigators studying central CO_2 chemosensitivity are fortunate to have numerous experimental preparations with which to conduct their studies. Unfortunately the findings across all preparations exhibit much variability and at times are inconsistent. With such

variable results comes much controversy. Although 5-HT neurons are thought by many to play a large role in central chemosensitivity, not everyone is completely convinced.

One argument that 5-HT neurons are not central CO_2 chemoreceptors is based on the assertion that 5-HT neurons do not have a large response to CO_2 *in vivo* (Guyenet et al., 2008). Guyenet et al. (2008) based this conclusion on recordings from rats under halothane anesthesia in which 5-HT neurons in the rostral ventrolateral medulla (RVLM) have a small hypercapnic response (Mulkey et al., 2004). However, other studies from unanesthetized animals have revealed that 5-HT neurons do indeed exhibit a robust response to hypercapnia (Veasey et al., 1997). It is challenging to explain why 5-HT neurons would be highly chemosensitive in brain slices and in culture (Richerson, 2004), but have little or no response *in vivo*. The most likely explanation for the small CO_2 response of 5-HT neurons in the RVLM seen *in vivo* by Mulkey et al. (2004) is the use of halothane (Massey et al., in review). Anesthesia is well known to have major effects on respiratory control. The experiments performed by Mulkey et al. (2004) were performed with halothane anesthesia, whereas other studies of chemosensitivity of 5-HT neurons *in vivo* were performed in unanesthetized animals (Veasey et al., 1997). This suggests that chemosensitivity of 5-HT neurons is blunted or masked by halothane anesthesia. A potential mechanism for this effect is that 5-HT neurons abundantly express TASK channels that are relatively inactive at rest, but are strongly activated by halothane (Sirois et al., 2000; Washburn et al., 2002). This activation could lead to a large current shunt, reducing the effect on membrane potential of changes in other currents, dampening or masking the response to hypercapnia.

In addition, many disagree about which experimental preparation is most beneficial/useful for studying central chemosensitivity. The perfused *in situ* brainstem preparation has been suggested to express a lower hypercapnic response than the *in vivo* preparation (Day and Wilson, 2009). We, in the laboratory of Dr. Michael Harris, strongly disagree with such a statement. Instead we propose that the two preparations are similarly sensitive, but that ventilatory sensitivities cannot be fully resolved *is situ*, from phrenic neurograms alone. We propose that intercostals contribute to enhanced tidal volume during respiratory stimulation. Thus, when intercostal recruitment is considered along with phrenic activity, the hypercapnic response of the *in situ* preparation may closely resemble that of the *in vivo* preparation (Harris et al., 2013).

1.13 Specific aim

The present dissertation describes studies developed to test the efficacy of an intermittent hypercapnia (IHc) protocol hypothesized to combat or reverse the abnormal response to hypercapnia associated with 5-HT dysfunction. Two levels of 5-HT dysfunction were induced, chronic partial and acute profound. Influences of the IHc protocol were assessed for duration of plasticity induced (acute vs. long-lasting) and whether or not there was a critical period in which the IHc protocol had its greatest effects. Induced plasticity was hypothesized to be due to an enhancement of GABA mechanisms and pharmacological antagonists were used to test this hypothesis. Although pharmacological antagonism was used as a preliminary assessment of mechanism of induced plasticity, the aim

of the present studies was not to determine the exact cellular/molecular mechanisms involved in this plasticity.

1.14 Experimental approach

The studies described in the current dissertation address the following questions: 1) Is the IHc-pretreatment protocol capable of overcoming the abolishment of hypercapnic responsiveness normally associated with the antagonism of ketanserin-sensitive mechanisms? 2) Is the IHc-pretreatment protocol capable of overcoming the reduced hypercapnic responsiveness induced by maternal tryptophan restriction? 3) Is the respiratory plasticity induced with the IHc protocol an acute or long-lasting effect? 4) Is there a critical period in which the IHc protocol has its greatest influence?

The *in situ* perfused decerebrate rat brainstem preparation was chosen as the experimental preparation to investigate the questions asked in this dissertation (Fig. 1.8). Used by many groups, this is a powerful preparation that is very useful for the study of the respiratory system (Paton, 1996; Toppin et al., 2007). Despite being decerebrate, the majority of the central nervous system remains intact, including the brainstem respiratory control network. An important advantage of the *in situ* preparation is the absence, after initial dissection, of anesthesia. In addition, the stability of this preparation allows for prolonged electrophysiological recordings. Furthermore because the animal is decerebrate and insentient, many pharmacological manipulations not possible *in vivo* are possible. Using suction electrodes, phrenic neurograms (activity of the phrenic nerve innervating the

diagram, which is the major pump muscle involved in respiration) were recorded. To ensure consistent recordings, glass micropipettes for suction electrodes were pulled to fit the size of the animal being used for the specific experiment being conducted. The response to a 4 % hypercapnic challenge (increase from 5 % CO_2 in perfusate to 9 %) was assessed for each animal. As central structures are more responsible for the response to CO_2, the preparations were perfused with a high level of oxygen (approximate P_{O_2} of 600 mmHg) to effectively eliminate any hypercapnic response from peripheral chemoreceptors (carotid and aortic bodies). This allowed us to investigate the influences of our IHc protocol on central mechanisms.

1.15 Major findings

Ours is the first reported observation that IHc-pretreatment can influence subsequent CO_2 chemoresponsiveness. I show that the plasticity induced by IHc-pretreatment is capable of overcoming the profound CO_2 chemoresponsive dysfunction produced by ketanserin treatment. With pharmacological antagonism, I show that the enhancement of CO_2 chemoresponsiveness is due to strengthening of bicuculline- and/or saclofen-sensitive $GABA_{A/B}$ mechanisms. In addition, IHc-pretreatment is capable of overcoming the mild CO_2 chemoresponsive dysfunction induced by dietary tryptophan restriction. Furthermore, I show that IHc-pretreatment induces long-lasting respiratory plasticity and strengthens bicuculline- and/or saclofen-sensitive $GABA_{A/B}$ contributions to the central

response to hypercapnia far into adult life (at least to P65). Lastly, I show that IHc-pre-treatment induces plasticity, sufficient to overcome disruption of ketanserin-sensitive mechanisms, regardless of the developmental period in which the IHc-pretreatment was administered. This indicates that there is no critical period for IHc-induced plasticity.

1.16 References

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1.17 Figures

See below where figures are displayed on full pages.

(a) (b)

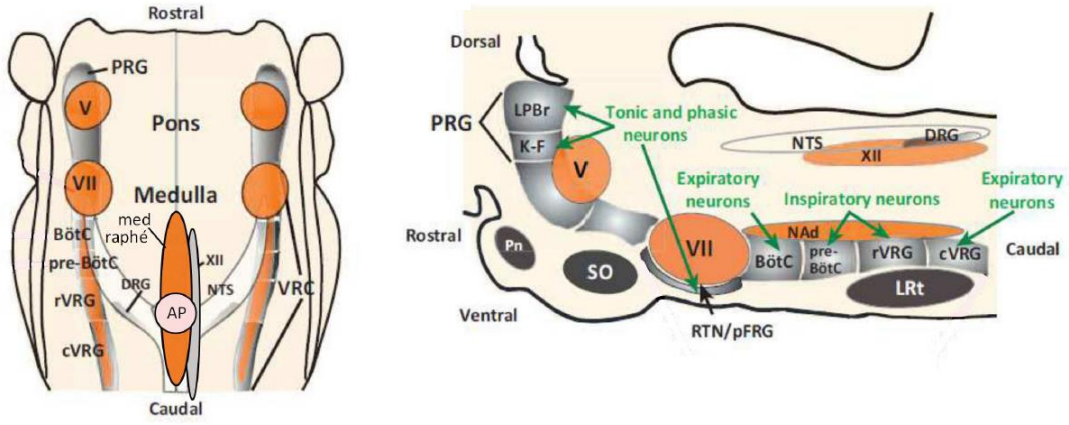

Figure 1.1 **Brainstem respiratory-related regions**. Neuroanatomy of the brainstem respiratory network arranged from the rostral pons to the caudal medulla. Horizontal from above (a) and parasagittal (b) views of the rat brainstem showing locations of the main groups of respiratory neurons in the mammalian central nervous system. Predominant locations of inspiratory, expiratory, tonic and respiratory-modulated (phasic) interneurons are indicated in (b). Other abbreviations: AP, area postrema; cVRG, caudal ventral respiratory group; med raphé, medullary raphé; Nad, nucleus ambiguous, dorsal division; Pn, ventral pontine nucleus; rVRG, rostral ventral respiratory group; SO, superior olivary complex; V, motor nucleus of the trigeminal nerve; XII, hypoglossal motor nucleus. Modified from Smith et al., 2013.

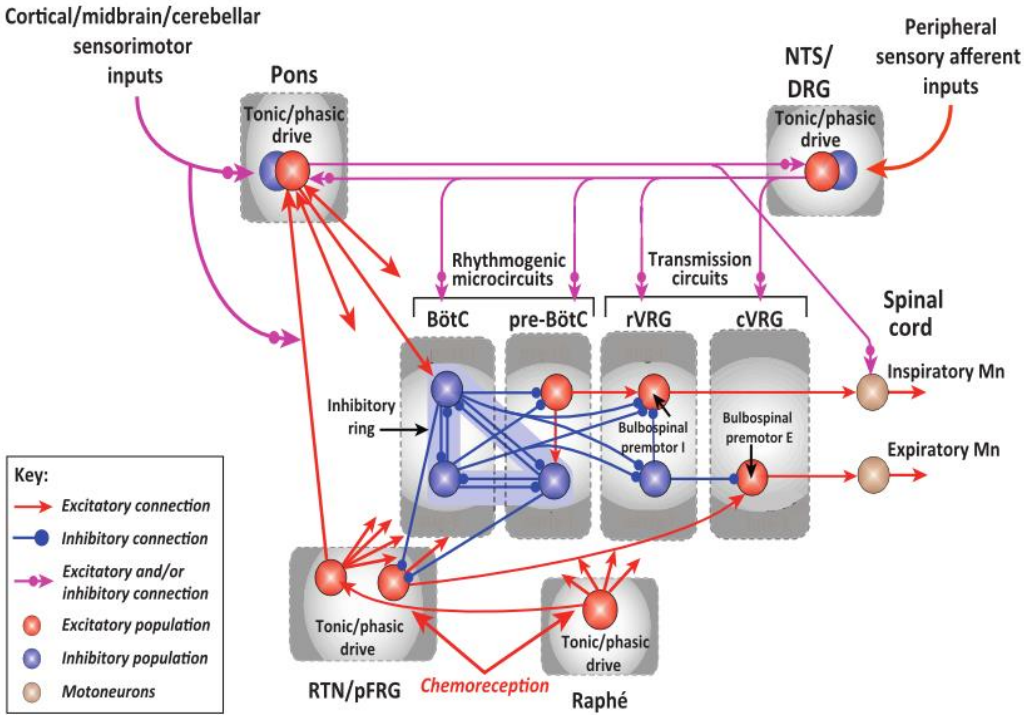

Figure 1.2 **Vital brainstem networks for breathing.** Schematic representation of brainstem regions and microcircuits involved in respiratory rhythm generation. The pre-Bötzinger complex (pre-BötC) and Bötzinger complex (BötC) are major components of the ventral respiratory column (VRC) generating multiple respiratory patterns as described in the text. This highly simplified diagram incorporates both excitatory and inhibitory regions, as well as the interaction between them. Neurons within the pre-BötC, BötC, rostral ventral respiratory group (rVRG) and caudal ventral respiratory group (cVRG) receive tonic, phasic or rhythmic excitatory drive from the pontine, dorsal respiratory group (DRG), retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) and raphé compartments. Drives from the latter two compartments are regulated in part by blood or brain CO_2 levels (chemoreception). Modified from Smith et al., 2013.

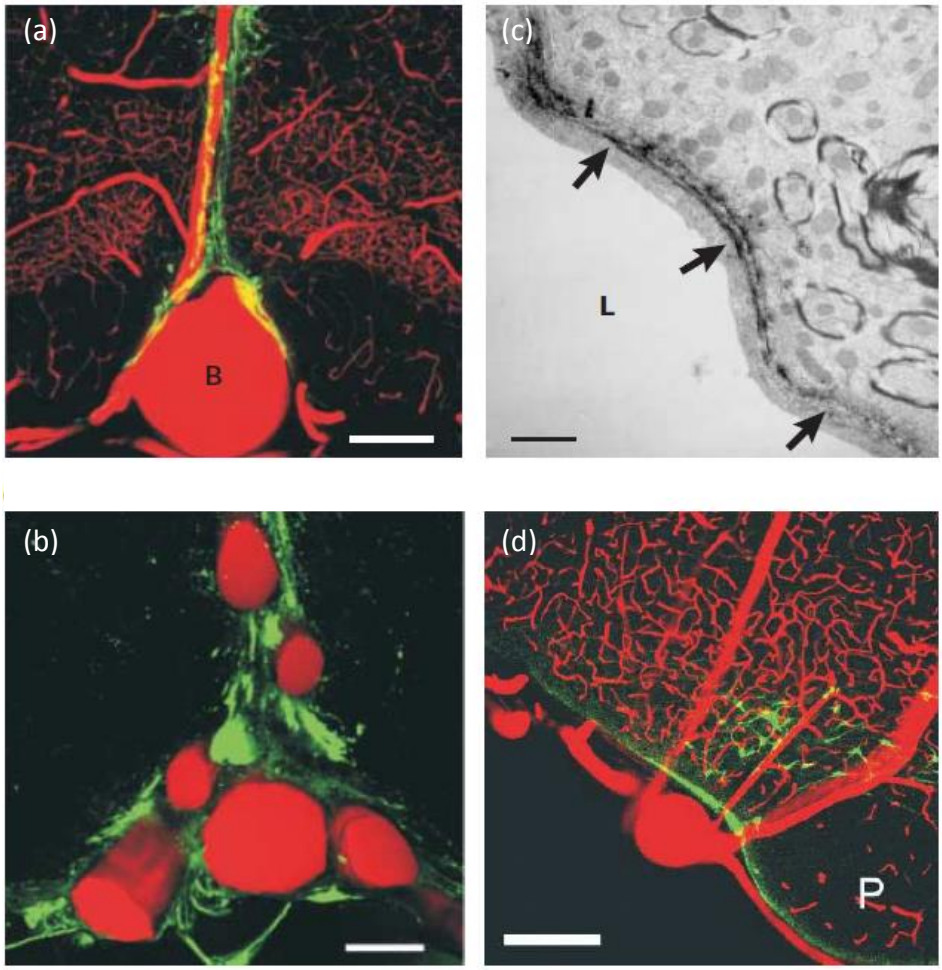

Figure 1.3 **Chemosensitive serotonergic neurons are closely associated with large medullary arteries.** (a): Serotonergic neurons (green and yellow) in the medullary raphé are closely associated with the basilar artery (B) and its main midline branches (red) as indicated by confocal imaging after immunohistochemistry for tryptophan hydroxylase. (b): Serotonergic neurons in the raphé pallidus also appear to be in close association with a dense plexus of arteries. (c): A dendrite of a serotonergic raphé neuron (arrows) follows the wall of a large midline artery, and comes within 0.5 μM of the lumen (L) as shown by

electron microscopy. (d): Serotonergic neurons on the ventrolateral surface of the medulla are also associated with large arteries. P, pyramidal tract. Scale bars 200 µm in (a) and (d), 50 µm in (b), and 1 µm in (c). Modified from Richerson, 2004.

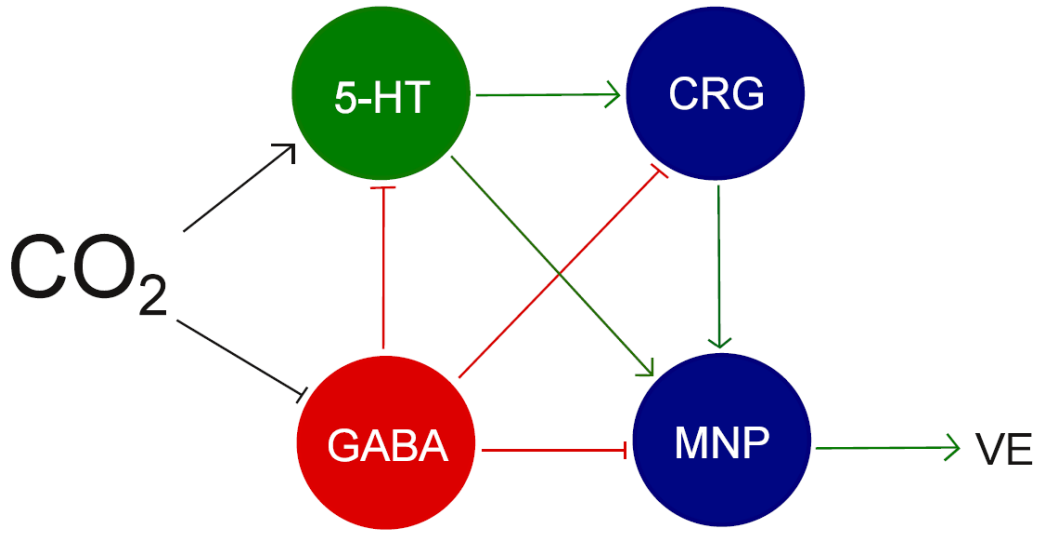

Figure 1.4 "**Push-pull" model of raphé contributions to the central response to hypercapnia.** The complex organization of central CO_2 chemoreception affords the potential for considerable plasticity, allowing for homeostatic regulation despite dysfunctional mechanisms, injury or disease. CO_2 not only activates excitatory 5-HT neurons in the medullary raphé, which are known to participate in respiratory control, but also deactivates inhibitory GABA neurons in this same region. This CO_2-mediated deactivation of the inhibitory GABA raphé pathway causes disinhibition, and resultant stimulation of breathing. Thus, the excitatory 5-HT and inhibitory GABA pathways are thought to behave in a "push-pull" manner to modulate ventilation (VE) via activity of central rhythm generators (CRG) and motor neuron pools (MNP).

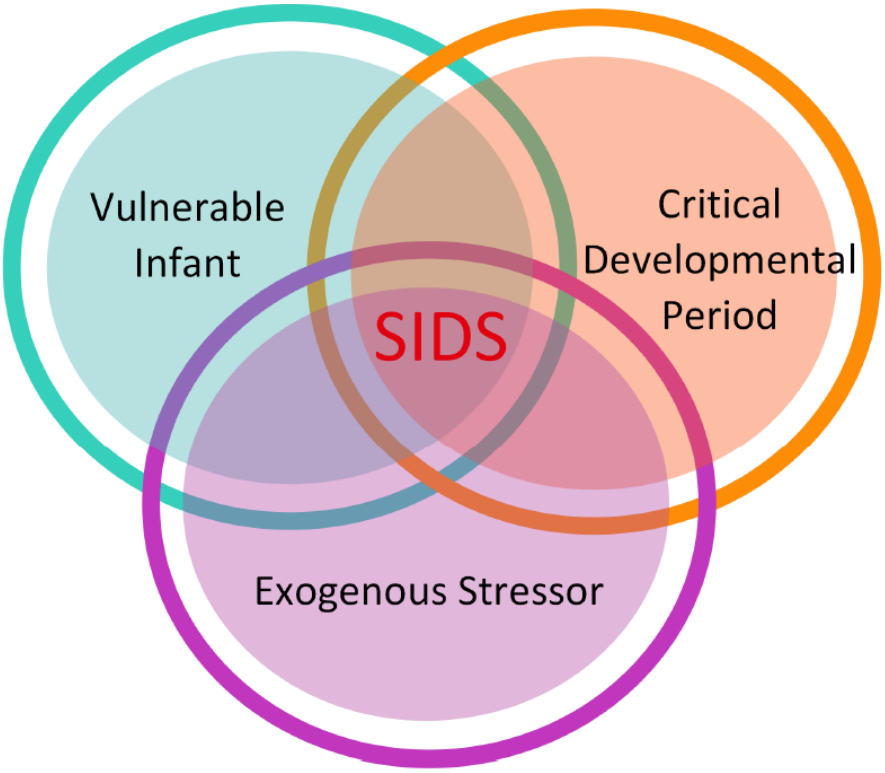

Figure 1.5 **Triple Risk Model of the Sudden Infant Death Syndrome.** According to this model, SIDS results when three factors simultaneously influence an infant: (a) an underlying vulnerability in the infant, (b) a critical developmental period and (c) an exogenous stressor, e.g., prone sleep position. In the context of the Triple Risk Model, the risk factors for SIDS can be divided into extrinsic and intrinsic categories. Extrinsic risk factors are acquired physical stressors related to the circumstances of death, e.g., prone sleep position. Such exogenous stressors are postulated to induce asphyxia, hypercapnia and hypoxia. Intrinsic risk factors, however, are associated with the underlying vulnerability/abnormality in the infant and increase the likelihood of SIDS by exacerbating this abnormality. Adapted from Filiano and Kinney, 1994.

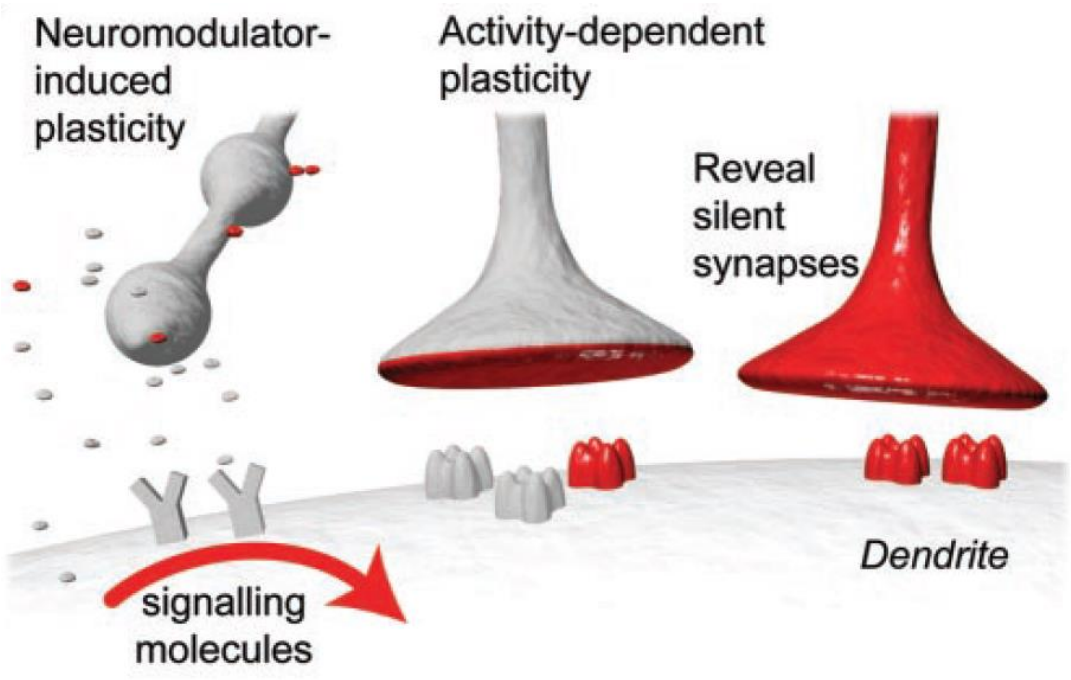

Figure 1.6 **Activity-dependent plasticity.** Plasticity is indicated by the red color. Synaptic plasticity may be induced by neuromodulators (neuromodulator-induced plasticity), which activate intracellular signaling molecules (red), secondarily altering the strength of other (glutamatergic or GABAergic) synaptic inputs. Activity-dependent plasticity may arise from coincident pre- and postsynaptic activity, thereby altering presynaptic transmitter release or postsynaptic receptor function in a manner similar to long-term potentiation and long-term depression. Silent synapses (anatomically present but functionally ineffective) may be revealed by neuromodulator or activity-dependent mechanisms. Modified from Mitchell and Johnson, 2003.

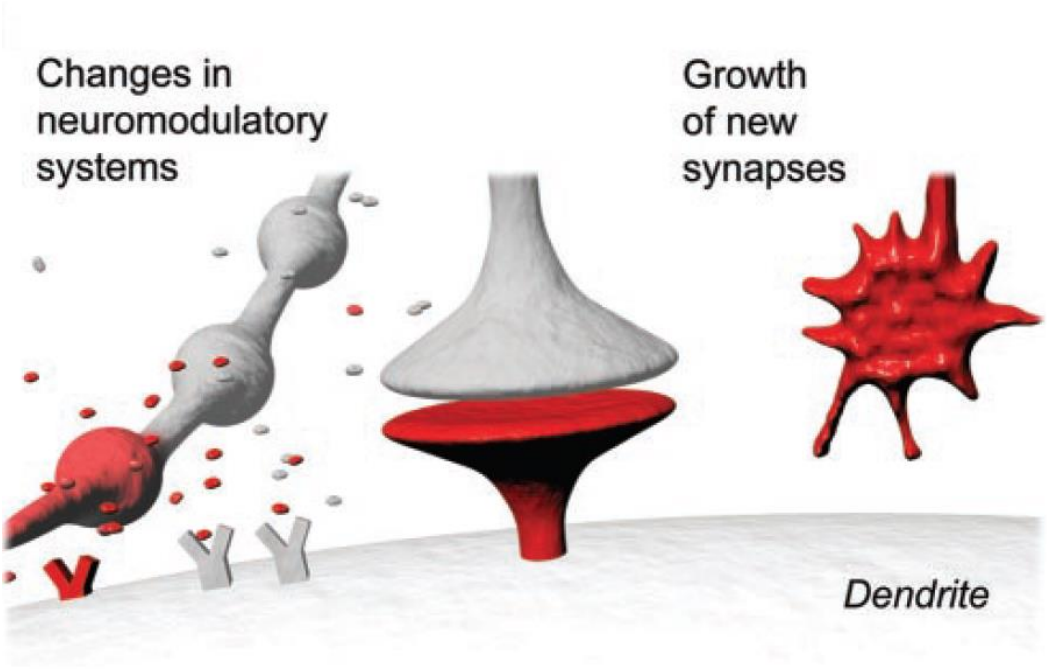

Figure 1.7 **Morphological changes underlying respiratory plasticity**. The structure/function of neuromodulatory systems may change, increasing or decreasing the capacity for neuromodulation. Neuron properties may change, such as size and shape of the dendrites or somata and density of dendritic spines. In addition, new synapses may be formed or pruned. Modified from Mitchell and Johnson, 2003.

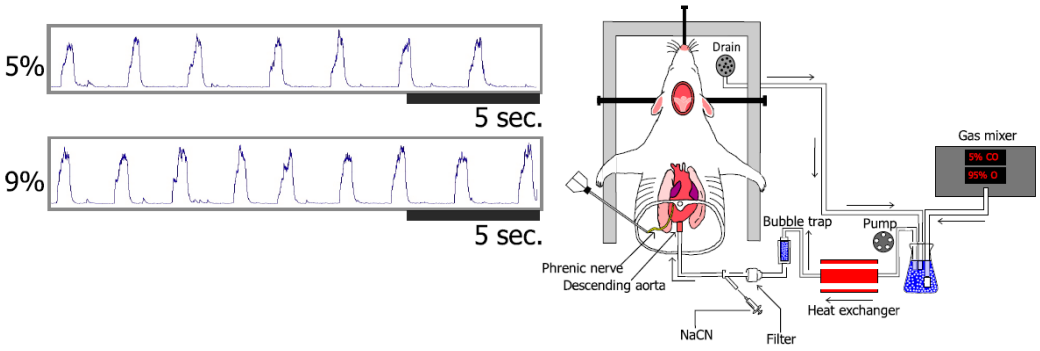

Figure 1.8 ***In situ* unanesthetized perfused decerebrate brainstem preparation for assessment of central chemosensitivity**. Central CO_2 chemosensitivity was assessed using an *in situ* unanesthetized perfused decerebrate brainstem preparation (Paton, 1996). Preparations were maintained with perfusate equilibrated with 5 % CO_2 in 95 % O_2, and exposed to a 5-min 4 %-hypercapnic challenge (9 % CO_2, balance O_2). Phrenic neurograms were recorded and analyzed for burst frequency, amplitude and the multiple (neural minute ventilation; NVE) to illustrate neural equivalents of breathing and sensitivity to the hypercapnic challenge.

Chapter 2

Intermittent hypercapnia enhances CO_2 responsiveness and overcomes ketanserin-induced dysfunction[1]

2.1 Abstract

Abnormal homeostatic responses to increased levels of CO_2 are associated with numerous dangerous or life-threatening disorders. We tested the hypothesis that postnatal exposure to mild intermittent hypercapnia (IHc) induces respiratory plasticity. Rats were exposed to an IHc protocol (or air control) for 5 days beginning at P12. We subsequently assessed CO_2 responsiveness using the *in situ* perfused brainstem preparation. Hypercapnia responses were determined in air or IHc-pretreated groups, both with and without pharmacological manipulation. Results confirmed CO_2 chemoresponsiveness of the experimental preparation and that ketanserin abolished this responsiveness in control groups. IHc-pretreatment enhanced CO_2 responsiveness. IHc-pretreatment maintained CO_2 responsiveness despite ketanserin treatment. We conclude that ketanserin-sensitive mechanisms are normally necessary for CO_2 chemoresponsiveness, that IHc-pretreatment enhances CO_2 responsiveness and that IHc-pretreatment induces non-ketanserin-sensitive contributions to CO_2 chemoresponsiveness. CO_2 responsiveness following IHc-pretreatment was absent if ketanserin was combined with bicuculline and saclofen, indicating that plasticity may depend on bicuculline- and/or saclofen-sensitive mechanisms.

[1] Mosher BP, Taylor BE, Harris MB. Intermittent hypercapnia enhances CO_2 responsiveness and overcomes ketanserin-induced dysfunction. *Respir. Physiol. Neurobiol., in review.*

We propose that IHc-induced plasticity could reduce the severity of reflex dysfunctions underlying pathologies associated with abnormal responses to hypercapnic conditions, possibly including the Sudden Infant Death Syndrome (SIDS)

2.2 Introduction

The brain's ability to detect and respond to changes in CO_2/pH (central CO_2 chemosensitivity) is a fundamental homeostatic process and essential for life. Specific brainstem neuron types responsible for central chemosensitivity *in vivo* are poorly understood and are the topic of ardent debate (Guyenet et al., 2005; Guyenet, 2008; Nattie and Li, 2009; Richerson et al., 2001). Central CO_2 chemoreception is likely best described as a complex system function that involves a limited but varied group of neuron types, brainstem sites and multiple neurotransmitter mechanisms (Feldman et al., 2003; Nattie, 2009; Nattie and Li, 2009; Putnam et al., 2004). It may be that different mechanisms contribute to sensitivity under different conditions. Organization of chemosensitivity as a system function affords the potential for considerable plasticity. Not only could a specific mechanism play a differential role in overall system sensitivity under specific conditions, the involvement of multiple mechanisms may allow homeostatic regulation despite partial dysfunction, injury or disease (Feldman et al., 2003). Thus, if one reflex mechanism is dysfunctional, alternative mechanisms may facilitate an appropriate response.

Medullary raphé neurons contribute to chemoresponsiveness. Located within the raphé are at least two neuronal subsets documented to possess intrinsic chemosensitivity

in vitro (Corcoran et al., 2009; Richerson, 2004; Richerson et al., 2005; Wang et al., 1998; Wu et al., 2008); acidosis-stimulated, serotonin(5-HT)-synthesizing neurons and acidosis-inhibited, γ-aminobutyric acid(GABA)-synthesizing neurons. We have developed a model describing raphé contributions to central chemosensitivity wherein both 5-HT and GABA mechanisms modulate ventilation to maintain tissue CO_2/pH through synaptic activation and disinhibition, respectively (Corcoran et al., 2008; Richerson, 1995). We have demonstrated that these cell types retain chemosensitivity in the intact and unanesthetized brainstem (Corcoran et al., 2008, 2013; Iceman et al., 2013). Hypercapnia stimulates 5-HT neurons, contributing to excitation of ventilation. Hypercapnia also inhibits normally inhibitory GABA neurons, stimulating ventilation through disinhibition (Hodges et al., 2004). Importantly, chemosensitivity must regulate ventilation in the hypocapnic range as well. We propose that hypocapnia/alkalosis not only inhibits 5-HT neurons reducing ventilatory stimulation, but also stimulates inhibitory GABA neurons to inhibit ventilation. So, although the ventilatory homeostatic response to hypercapnia is normally primarily mediated by 5-HT-synthesizing neurons (Corcoran et al., 2008), it may be possible that strengthening alternative mechanistic pathways could preserve responsiveness to a hypercapnic challenge despite a dysfunction in 5-HT mechanisms.

Ventilatory CO_2 chemoresponsiveness is abolished by acute disruption of 5-HT mechanisms (Corcoran et al., 2013). However, genetically modified mice chronically lacking 5-HT neurons retain partial CO_2 responsiveness (Hodges et al., 2008). We speculate that although 5-HT mechanisms are normally critical, plasticity in response to a chronic

absence of 5-HT neurons can accommodate appropriate CO_2 chemoresponsiveness by inducing alternative mechanisms. We propose that plasticity could be induced to precondition the system and enhance other chemosensory mechanisms. We tested the hypothesis that preconditioning with intermittent hypercapnia (IHc) during postnatal development induces plasticity, and that this plasticity compensates for the loss of normally critical 5-HT mechanisms. Furthermore, based on our model of 5-HT- and GABA-mediated contributions to the central CO_2 response, we hypothesized that plasticity results from enhancement of GABA-mediated mechanisms.

Abnormalities in 5-HT brainstem nuclei are associated with infant vulnerability to the Sudden Infant Death Syndrome (SIDS), and that vulnerability may result in part from CO_2 chemoresponsive dysfunction (Cummings et al., 2009; Duncan et al., 2010; Hodges and Richerson, 2010; Kinney et al., 2009; Paterson et al., 2006; Richerson, 2004). If induced plasticity is sufficient to overcome or reverse ventilatory CO_2 chemoresponsive dysfunctions similar to those thought to contribute to SIDS, then interventions that induce plasticity could be therapeutic in augmenting CO_2 chemoresponsiveness in vulnerable individuals.

2.3 Methods

Experimental groups: All experiments were done in accordance with the guidelines of the "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health and were approved by the University of Alaska Fairbanks (UAF) Institutional Animal Care

and Use Committees. Nineteen naïve rat dams received normal rat chow and water *ad libitum*, and were bred with 19 males. Sprague-Dawley rats were used in all experiments (Simonson Laboratories). Resulting pups also received food and water *ad libitum* and were housed and maintained in the UAF Animal Care Facility on a 12 h light/dark cycle, at a room temperature of 21 °C. A total of 112 animals from both sexes were used in these studies.

Gas pretreatments: Rat pups were exposed to intermittent hypercapnia (IHc; 8 consecutive cycles of 5 min 5 % CO_2: balance air, followed by 10 min air) or constant normocapnia as a control (Nc; Type 1-Grade D air only, as a treatment sham) each day for 5 consecutive days beginning at post-natal day 12 (P12). Entire litters were randomly assigned to either IHc- or Nc-pretreatment. Because related litter-mates, rather than randomly assigned individuals, were used as experimental subjects, multiple litters (2 to 5) received each combination of gas and pharmacological treatments. During gas treatments, dams were separated from pups and the home cage was transported to a procedure room adjacent to the animal holding area. Cages were fitted with an airtight lid with a gas inflow and outflow. In this manner, litters (ranging from 3 to 15 pups) were exposed to room temperature inlet gas, supplied at 10 l/min through a countercurrent heat exchanger. The gas outlet was connected to 1 m of 5.5 mm internal diameter tubing to prevent room air infiltration without creating substantial positive pressure within the chamber. For intermittent gas exposure, a programmable digital timer and solenoid valve automatically switched inlet gases between the two sources. This apparatus produced a 10-

min period of normocapnia followed by 8 repeated cycles of 5-min hypercapnia (or normocapnia in controls) and 10-min normocapnia. Valve cycling was monitored using a computerized data acquisition system (LabChart 7, ADInstruments). Pilot studies indicated that CO_2 levels in the enclosure equilibrated with inlet gas concentrations within 90 s. Chamber gas was not monitored during these treatments. After this exposure protocol, cages were removed from the enclosure, dams were returned to pups in the home cage and cages were returned to the adjacent housing facility. In no cases did pup abandonment occur following these brief maternal separations, and patterns and durations of maternal separation were equal between IHc and Nc treatment groups. Exposure protocols were repeated on 5 consecutive days, at approximately the same time each day.

In situ assessment of CO_2 chemoresponsiveness: At least 7 days following IHc- or Nc-pretreatments, hypercapnic ventilatory responsiveness was assessed using the *in situ* arterially perfused brainstem preparation as previously described (Corcoran et al., 2013; Toppin et al., 2007, after Paton, 1996). Briefly, animals were anesthetized with isoflurane (5 %, vaporized in 100 % O_2) and pretreated with heparin sodium (500 units, I.P.). Animals were bisected subdiaphragmatically and submerged in an ice-chilled artificial cerebral spinal fluid (aCSF) containing (mM in H_2O): $MgSO_4 \cdot 7H_2O$, 1; $NaH_2PO_4H_2O$, 1.25; KCl, 4; $NaHCO_3$, 24; NaCl, 115; D-glucose, 10; $CaCl_2 \cdot 2H_2O$, 2). The forebrain rostral to the colliculi was removed by aspiration, and fur, skin and viscera were removed. The diaphragm was separated from the body wall with care taken to ensure integrity of the phrenic nerve. The preparation was moved to the recording station where the descending aorta was

cannulated using a double-lumen catheter (Ø 1.25 mm, Braintree Scientific) and perfused retrogradely from a reservoir containing 350 ml aCSF (with 13 g/l ficoll 70, Sigma, added as an osmotic agent). The perfusate was first equilibrated with 95 % O_2-5 % CO_2 (normocapnia, pH 7.4, P_{CO_2} of 33 mmHg). Equilibration mixtures were produced from O_2 and CO_2 using a precision gas mixer (GSM3, CWE) and verified with a CO_2 analyzer (CD-3A, Applied Electrochemistry). Normocapnic (baseline) conditions approximated normocapnic plasma *in vivo*. Lacking hemoglobin, solution hyperoxia ($P_{O_2} \approx 600$ mmHg) was necessary to maintain O_2 content sufficient to meet tissue metabolic demands. This unavoidable hyperoxia was constant under all conditions. Perfusate was warmed to 32 °C, and circulated through a bubble trap and particle filters (25 µm, 45 µm; Millipore) prior to entering the aorta. Perfusate passing through the animal was collected and recycled to the reservoir. The neuromuscular blocker gallamine triethiodide (20 mg/l), and the vaso-constricting hormone vasopressin (5 µM) were added to the perfusate. The aortic perfu-sion pressure was adjusted to 70-80 mmHg using an adjustable perfusate bypass valve and a bolus of sodium cyanide (50 µl 0.1 % solution) was injected into the perfusate line to transiently stimulate peripheral chemoreceptors (Dutschmann et al., 2000). After par-tial pulmonectomy, the phrenic nerve was exposed and aspirated into a glass capillary suction electrode pulled to a diameter that ensured adequate seal on the nerve.

The signal was amplified (×10,000; DAM50, WPI) and filtered (band-pass 300 Hz – 1 kHz). Using a computerized data acquisition system (Powerlab, ADInstruments), data were digitized at 1 k Samples/s and digitally integrated through full wave rectification and

50 ms moving average on a duplicate channel. Phrenic burst frequency (f, bursts/min) was determined by counting the number of phrenic bursts occurring during 60 s of normocapnia immediately preceding the gas challenge, the final 60 s of hypercapnia, and 60 s during normocapnic recovery 5 min after a return to baseline. Phrenic burst amplitude was derived from the mean integrated peak height of all bursts within these 60 s periods, expressed in arbitrary units within each preparation and as a proportional change within a preparation in response to the gas challenge. Neural minute ventilation (NVE) was calculated as the product of burst frequency and amplitude, again expressed in arbitrary units and as a proportional change with treatment (Eldridge, 1971).

Gas Challenges: Preparations were maintained on normocapnic perfusate for at least 1 h. For the gas challenge, gas equilibrating the perfusate was switched in sequence to 5-min periods of hypocapnia (96.5 % O_2-3.5 % CO_2; pH 7.5; P_{CO_2} ≈ 23 mmHg) followed by 5-min periods of hypercapnia (91 % O_2-9 % CO_2; pH 7.2; P_{CO_2} ≈ 60 mmHg) and subsequently returned to baseline normocapnia. The hypercapnic challenge conditions approximated those occurring during a 4 % increase in inspired CO_2.

Pharmacological challenges: Preparations were tested either without further pharmacological manipulation, or with the addition of specific antagonists to disrupt particular neurotransmitter signaling mechanisms. Pharmacological agents were added after the 60-min normocapnic period, which was maintained for an additional 10-min prior to gas challenge. In one series, the 5-HT_2 receptor antagonist ketanserin tartrate (5 μM,

Sigma) was administered to determine how subsequent responsiveness to CO_2 was affected by impairment of ketanserin-sensitive processes. We have previously shown that a ketanserin-sensitive mechanism is important for CO_2 chemoresponsiveness in this system (Corcoran et al., 2013). In a second series, the $GABA_A$ and $GABA_B$ receptor antagonists bicuculline (20 μM, Sigma) and saclofen (20 μM, abcam) were administered to determine to what degree the hypercapnic ventilatory response was due to bicuculline- and saclofen-sensitive mechanisms. In a third series, a combination of ketanserin and bicuculline and saclofen was administered to assess the influence of combined antagonism of ketanserin-sensitive and bicuculline- and saclofen-sensitive processes on hypercapnic response.

Data and statistical analyses: Ventilatory parameters were quantified from the integrated neurograms. Frequency (f; the number of bursts per unit time), amplitude (the mean integrated peak amplitude of bursts), and a neural correlate of minute ventilation (NVE; frequency·amplitude) were calculated from periods of normocapnia or hypercapnia. Hypercapnic responsiveness was determined from ventilatory parameters recorded during baseline normocapnia and hypercapnia. This was quantified by deriving a relative hypercapnic response, calculated by expressing the difference in parameter values between normocapnia and hypercapnia, normalized as a percentage relative to values occurring with normocapnia prior to gas challenge (responsiveness = [value during hypercapnia – value during normocapnia] / value during normocapnia).

Data Parsing: We and others have demonstrated that the *in situ* rat brainstem preparation exhibits clear responsiveness to hypercapnia and provides an appropriate model for the study of mechanisms contributing to chemosensitivity (Corcoran et al., 2013; Day and Wilson, 2007; Iceman et al., 2013; Toppin et al., 2007). These prior studies have illustrated that the response is most clearly manifest in changes in burst frequency and the index of minute ventilation, NVE. Our pilot investigations suggested the possibility that preparations exhibiting a relatively high initial burst frequency have a lower apparent responsiveness to hypercapnia, which suggests a frequency limitation. The present study was designed to investigate mechanisms contributing to CO_2 responsiveness, necessitating a focus on responsive preparations free of potential confounding factors.

To remove the potential confounding influence of dampened CO_2 chemoresponsiveness in preparations with elevated baseline burst frequencies, we subjected the data set to the following parsing protocol. In preparations exposed to neither pharmacological manipulation nor IHc-pretreatment, we plotted the relationship between hypercapnic responsiveness and initial burst frequency and determined a linear regression ($P < 0.001$; $R^2 = 0.74$) described by the equation: hypercapnic responsiveness (HcR) = 192 – 3.45 (initial frequency). The highest responsiveness observed in any of these preparations was an 84.6 % increase. From this regression, we determined the initial frequency associated with the half-maximum CO_2 responsiveness. We then used this value as a threshold to identify preparations in which high initial burst frequency could confound expression of CO_2 chemoresponsiveness. Preparations having a normocapnic initial burst frequency above

this threshold were omitted from all portions of analysis. When so parsed, 17 of 112 preparations (15 %) were removed from analyses; a total of 95 preparations contributed to the analyzed data set.

One-way repeated-measures analysis of variance (RM-ANOVA) was used to compare parameter values before and during hypercapnia for each drug treatment (no drugs; ketanserin alone; bicuculline + saclofen; ketanserin + bicuculline + saclofen), which quantified hypercapnic responsiveness for ventilatory parameters under each drug treatment. One-way ANOVA was used to compare the effect of drug treatment on the relative hypercapnic response in each parameter. Values reported in the text are mean ± standard error. To compare the mean hypercapnic responsiveness for each parameter between groups, a Tukey post-hoc analysis was used following a significant ANOVA.

2.4 Results

Burst discharge recordings: All our recorded phrenic neurograms displayed a "eupneic" pattern (Fig. 2.1) characteristic of this preparation (Eldridge, 1971; St.-John and Paton, 2000), and displayed this pattern throughout normocapnia, hypercapnia and recovery from normocapnia, and drug treatment. Preparations without pharmacological manipulation derived from pups that were Nc-pretreated (sham air exposures), had a mean normocapnic burst frequency of 20.7 ± 1.26 bursts/min.

Response to hypercapnia: In preparations derived from pups pretreated with Nc, mean f and NVE increased during hypercapnia by 22 ± 5.0 % (Fig. 2.2) and 27 ± 14 % (Fig.

2.3), respectively. One-way RM-ANOVA indicated that these hypercapnia-induced changes were significant ($P < 0.001$ for f; $P < 0.05$ for NVE). No significant responsiveness was resolved for burst amplitude alone ($P = 0.62$). Nc-pretreated preparations that received ketanserin had no significant response to hypercapnia (non-significant increases in f = 3.4 ± 7.5 %, $P = 0.61$, Fig. 2.2; NVE = 5.6 ± 11.0 %, $P = 0.58$; Fig. 2.3; amplitude = -0.3 ± 6.0 %, $P = 0.83$, Fig. 2.4). Statistically, the absent responsiveness with ketanserin was different from the responsiveness present without ketanserin treatment in Nc-pretreated preparations (frequency, $P < 0.05$, Fig. 2.2). In preparations derived from pups pretreated with Nc, and treated with bicuculline and saclofen, f and NVE increased during hypercapnia by 31 ± 10 % ($P < 0.05$; Fig. 2.2) and 61 ± 10 % ($P < 0.05$; Fig. 2.3), but did not increase amplitude 24 ± 9.8 ($P = 0.11$; Fig. 2.4) respectively. The hypercapnic responsiveness of preparations derived from Nc-pretreated pups with bicuculline and saclofen was no different than the responsiveness for preparations derived from pups pretreated with Nc that did not receive pharmacological manipulation ($P = 0.43$ for f; $P = 0.28$ for NVE; $P = 0.62$ for amplitude). As ketanserin treatment alone abolished CO_2 chemoresponsiveness in Nc-pretreated preparations, bicuculline and saclofen were not co-applied with ketanserin.

In preparations from pups pretreated with IHc, mean f and NVE increased during hypercapnia (increases of 38 ± 11 %, $P < 0.001$, Fig. 2.2; and 130 ± 58 %, $P < 0.001$, Fig.

2.3; respectively), but burst amplitude did not ($P = 0.68$; Fig 2.4). Hypercapnic responsiveness as measured by NVE was significantly greater in preparations from IHc-pretreated pups than those from Nc-pretreated pups ($P \leq 0.05$, Fig. 2.3).

Unlike in Nc-pretreated pups, in preparations derived from pups pretreated with IHc, f and NVE increased in response to hypercapnia under ketanserin treatment (f = 28 ± 3 %, $P < 0.001$, Fig. 2.2; NVE: 29 ± 5 %, $P < 0.001$, Fig. 2.3). This responsiveness was less than that of preparations derived from IHc-pretreated pups without ketanserin treatment (NVE, $P < 0.05$, Fig. 2.3) but was no different than that of Nc-pretreated preparations without ketanserin (frequency, $P = 0.25$; NVE, $P = 0.90$).

When bicuculline, saclofen and ketanserin were co-applied in preparations derived from IHc-pretreated pups, none of the parameters (frequency, NVE or amplitude) indicated a response to hypercapnia. This responsiveness was less than the responsiveness present subsequent to ketanserin treatment alone in IHc-pretreated preparations (frequency, $P < 0.001$, Fig. 2.2; NVE: $P < 0.01$, Fig. 2.3), and equal to that of Nc-pretreated preparations treated with ketanserin alone (frequency, $P = 0.28$, Fig. 2.2; NVE: $P = 0.47$, Fig. 2.3). Hypercapnic challenge failed to increase phrenic burst amplitude in any group. Additionally, there was no difference between the burst amplitude responses to hypercapnia between groups (Fig. 2.4).

The *in situ* preparation is responsive to hypercapnia under control (Nc-pretreated) conditions, and this response is dependent on ketanserin-sensitive mechanisms, but not

on bicuculline- and saclofen-sensitive mechanisms. The hypercapnic response was enhanced by IHc-pretreatment, and a portion of the hypercapnic response was retained despite removal of ketanserin-sensitive mechanisms. Retained chemoresponsiveness was dependent on bicuculline- and/or saclofen-sensitive mechanisms.

2.5 Discussion

Our study assessed the effect of pretreatment with intermittent hypercapnia during postnatal development on CO_2 chemoresponsiveness. We tested the hypothesis that postnatal IHc-pretreatment induces respiratory plasticity. Specifically, we assessed if IHc-pretreatment would enhance hypercapnic responsiveness, and if this enhancement would compensate for the compromised responses induced by ketanserin treatment. Furthermore, we tested the hypothesis that bicuculline- and saclofen-sensitive mechanisms contribute to this plasticity.

Our results confirm that the rat *in situ* perfused brainstem preparation does exhibit a CO_2 response (Corcoran et al., 2013; Day and Wilson, 2007). We also confirmed previous findings that ketanserin-sensitive mechanisms are critical to CO_2 chemoresponsiveness (Corcoran et al., 2013), as ketanserin treatment alone abolishes CO_2 responsiveness in preparations derived from Nc-pretreated rats. The primary influence of ketanserin, in respiratory control mechanisms, is as a post-synaptic 5-HT_2 receptor antagonist (Awouters, 1985, Corcoran et al., 2013). Previous studies investigating CO_2 chemoresponsiveness using the *in situ* preparation have used ketanserin to determine the role of post-

synaptic 5-HT_2 receptors (Corcoran et al., 2013). As ketanserin not only blocks 5-HT_2 receptors but also alpha-1 adrenergic (Hoyer et al., 1987) and histamine H1 receptors (Borroto-Escuela et al., 2014), as well as 5-HT_7 and dopamine D1 and D2 receptors with limited affinity (Shen et al., 1993; Wouters et al., 1985), Corcoran et al. (2013) acknowledge that the disrupted response to the hypercapnic challenge may have resulted from a dependence of CO_2 chemoresponsiveness on alpha-1 adrenergic, histamine H1 or 5-HT_7 receptor activation. However, the primary outcome of ketanserin administration was similar to that resulting from the administration of the 5-HT_{1A} receptor agonist (R)-(+)-8-hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT; Corcoran et al., 2013) which is commonly used in respiratory studies to inhibit 5-HT neuron transmitter release via activation of hyperpolarizing 5-HT_{1A} autoreceptors (St. John and Paton, 2000). In addition, a similar outcome to ketanserin was also observed following application of the mixed 5-$HT_{1,2}$ receptor antagonist methysergide (Harris et al., 2003). Thus, the most likely conclusion is that elimination of the hypercapnic ventilatory response by ketanserin was due to disruption of 5-HT processes, and that ketanserin-sensitive influences illustrate 5-HT contributions to hypercapnic responsiveness. We attribute our observation of ketanserin-sensitive CO_2 chemoresponsiveness to suggest that 5-HT neurotransmission is critical for CO_2 chemosensitivity in this experimental system (Corcoran et al., 2013).

Despite this apparent critical role of 5-HT mechanisms, central CO_2 chemosensitivity is a complex system function that involves multiple neuron types, brainstem sites, and neurotransmitter mechanisms (Feldman et al., 2003; Nattie, 2009; Nattie and Li, 2009;

Putnam et al., 2004). Diversity and redundancy in such a system function allows for plasticity and homeostatic regulation despite dysfunction in any one pathway (Feldman et al., 2003).

We illustrate that plasticity induced through IHc-pretreatment can overcome the CO_2 chemoresponse impairment normally associated with disruption of ketanserin-sensitive mechanisms. We propose that chemosensory plasticity can be induced with preconditioning to enhance multiple chemosensory mechanisms, specifically facilitating the recruitment of non-ketanserin-sensitive mechanisms to preserve CO_2 chemoresponsiveness even under circumstances in which ketanserin would normally eliminate the hypercapnic response.

Under normocapnic conditions, neuroventilatory parameters in preparations pretreated with IHc were comparable to those of preparations derived from Nc-pretreated pups. Preconditioning with IHc, however, greatly augmented CO_2 chemoresponsiveness. When compared to Nc-pretreated preparations, CO_2 chemoresponsiveness was increased 73 % for frequency and 381 % for NVE, in IHc-pretreated preparations.

Ours is the first reported observation that IHc-pretreatment can influence subsequent CO_2 chemoresponsiveness. In these experiments, IHc-pretreatments were initiated on P12 and continued daily through P16. Protocols were designed to administer cycles of mild hypercapnia to pups, without altering maternal conditions and while limiting maternal separation. This developmental period was chosen to follow the apparent critical period proximal to P12 where rat CO_2 chemosensitivity is altered (Putnam et al., 2005;

Wong-Riley and Liu, 2005, 2008), and the developmental period beyond which CO_2 chemosensitive 5-HT neurons have been identified *in vitro* (Wang and Richerson, 1999). Prior attempts to influence hypercapnic responsiveness showed no influence when a comparable IHc protocol was conducted between P7 and P14 (Steggerda et al., 2009). It is likely that the developmental timing and/or nature of the IHc stimulus influences resultant plasticity.

We attribute the lack of hypercapnic response after application of ketanserin to disruption of normally critical 5-HT mechanism. We attribute the retention of relatively normal CO_2 chemoresponsiveness, overcoming the expected ketanserin-mediated abolishment of CO_2 chemoresponsiveness, to plasticity imparted by the IHc-pretreatment. We conclude that IHc-pretreatment induced a ketanserin-insensitive mechanism not obviously critical to hypercapnic responsiveness. This plasticity appears sufficient to overcome what is normally a complete lack of hypercapnic responsiveness associated with ketanserin administration.

The organization of CO_2 chemosensitivity as a system function grants the potential for considerable plasticity. Not only could a particular mechanism play a differential role in overall system sensitivity under particular conditions, the involvement of multiple mechanisms may allow homeostatic regulation despite partial dysfunction, injury, or disease (Feldman et al., 2003). Thus, if one reflex mechanism is dysfunctional, alternative mechanisms may accommodate an appropriate response. Our observations indicate that plasticity initiated with IHc-pretreatment resulted in CO_2 chemoresponsiveness mediated

by mechanisms other than the ketanserin-sensitive mechanisms normally critical for CO_2 responsiveness. As such, we investigated whether IHc-induced plasticity was enhancing an alternate non-ketanserin-sensitive mechanism.

We have suggested that interactions between chemosensory mechanisms mediated either by 5-HT- or GABA-synthesizing neurons contribute to CO_2 chemosensitivity (Iceman et al., 2010; Richerson et al., 2001, 2005). As such, we predicted that the ketanserin-insensitive chemoresponsiveness induced by IHc-pretreatment was due to an enhancement of GABA-mediated mechanisms (Corcoran et al., 2008; Richerson, 1995). To broadly investigate the contribution of GABA mechanisms, we tested the combined influences of $GABA_A$ and $GABA_B$ receptor antagonists bicuculline and saclofen. Bicuculline and saclofen treatment had no influence on CO_2 chemoresponsiveness in Nc-pretreated preparations, indicating that bicuculline- and saclofen-sensitive $GABA_{A/B}$ receptor-mediated processes are not obviously critical to CO_2 chemoresponsiveness under normal conditions. Bicuculline- and saclofen-sensitive processes may not normally be necessary, and other processes may be sufficient, for hypercapnic responsiveness.

We assessed whether or not the ketanserin-insensitive CO_2 responsiveness present in IHc-pretreated preparations was mediated by bicuculline/saclofen-sensitive mechanisms. Although IHc-pretreated preparations maintained CO_2 chemoresponsiveness when treated with ketanserin alone, CO_2 responsiveness was absent in preparations with combined ketanserin, bicuculline and saclofen treatment. These results suggest that

the plasticity induced by IHc-pretreatment, responsible for ketanserin-insensitive CO_2 responsiveness, was induced through bicuculline- and/or saclofen-sensitive processes not normally critical for CO_2 chemoresponsiveness.

2.6 Conclusion

Our findings are consistent with considerations of central CO_2 chemosensitivity as a system function involving multiple neuron types and neurotransmitter mechanisms. We propose that 5-HT and GABA mechanisms contribute to CO_2 chemosensitivity. We confirm the finding that, in otherwise untreated preparations, ketanserin-sensitive mechanisms are important for the ventilatory response to CO_2. We show that IHc-pretreatment induces plasticity such that CO_2 chemoresponsiveness is maintained despite removal of otherwise critical ketanserin-sensitive mechanisms, and that this plasticity depends on bicuculline- and/or saclofen-sensitive mechanisms that are not obviously critical to CO_2 chemoresponsiveness under normal conditions.

Abnormal homeostatic responses to increased levels of CO_2 are associated with numerous dangerous or life-threatening disorders. Serotonin dysfunction likely contributes to a number of pathologies, including the Sudden Infant Death Syndrome (SIDS), by compromising homeostatic reflexes such as hypercapnic chemoresponsiveness (Kinney et al., 2009). It may be that IHc-induced plasticity to enhance the contributions of other neurotransmitter pathways to CO_2 chemoresponsiveness could enhance homeostatic reflex efficacy and potentially reduce vulnerabilities to conditions such as SIDS. A number

of critical factors remain unknown. These include: identification of the exact mechanisms underlying bicuculline- and saclofen-sensitive chemosensory plasticity; changes in ketanserin-sensitive mechanisms with IHc-pretreatment; the degree to which IHc-pretreatment can overcome chronic chemosensory dysfunctions similar to those associated with various pathologies; potential developmental sensitivities to IHc-induced plasticity; and the longevity of this plasticity. The clear potential to induce chemosensory plasticity, however, provides possible targets for therapeutic intervention to reverse or offset chemosensory dysfunction.

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2.8 Figures

See below where figures are displayed on full pages.

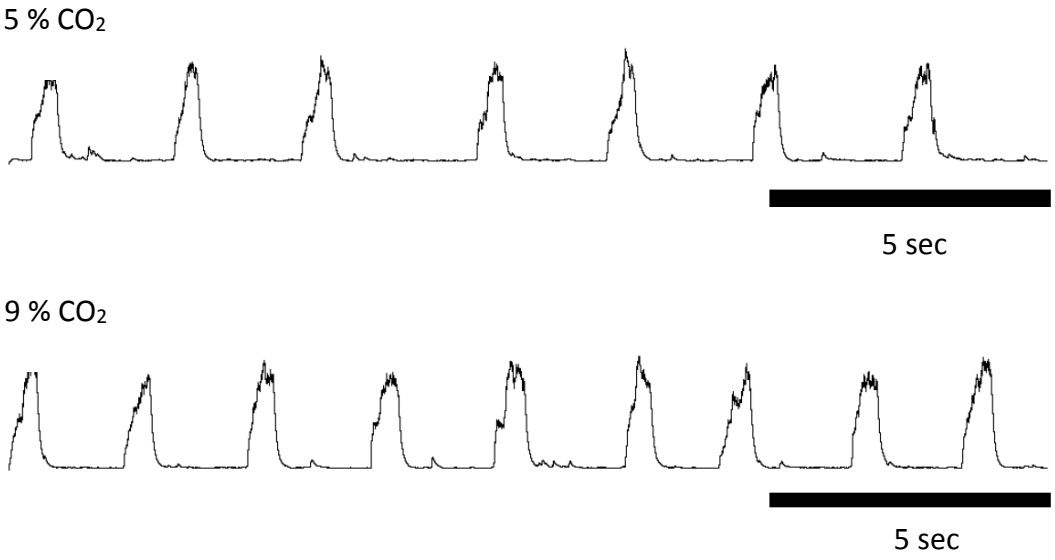

Figure 2.1 **Eupneic phrenic ventilatory burst *in situ***. Typical eupneic integrated phrenic neurogram recording with ventilatory bursts representing the neural correlate of ventilation (Eldridge, 1971; St.-John and Paton, 2000). When the *in situ* preparation was exposed to perfusate containing 9 % CO_2 (4 % hypercapnic challenge; bottom panel) there was an increase in both frequency and NVE (frequency*amplitude) of ventilatory events compared to 5 % CO_2 exposure (normocapnia; top panel). Both panels are representative records of the last minute of the indicated CO_2 treatment. Tracings from a preparation derived from a pup that received Nc-pretreatment (sham treatment) and no pharmaco-logical manipulations are shown.

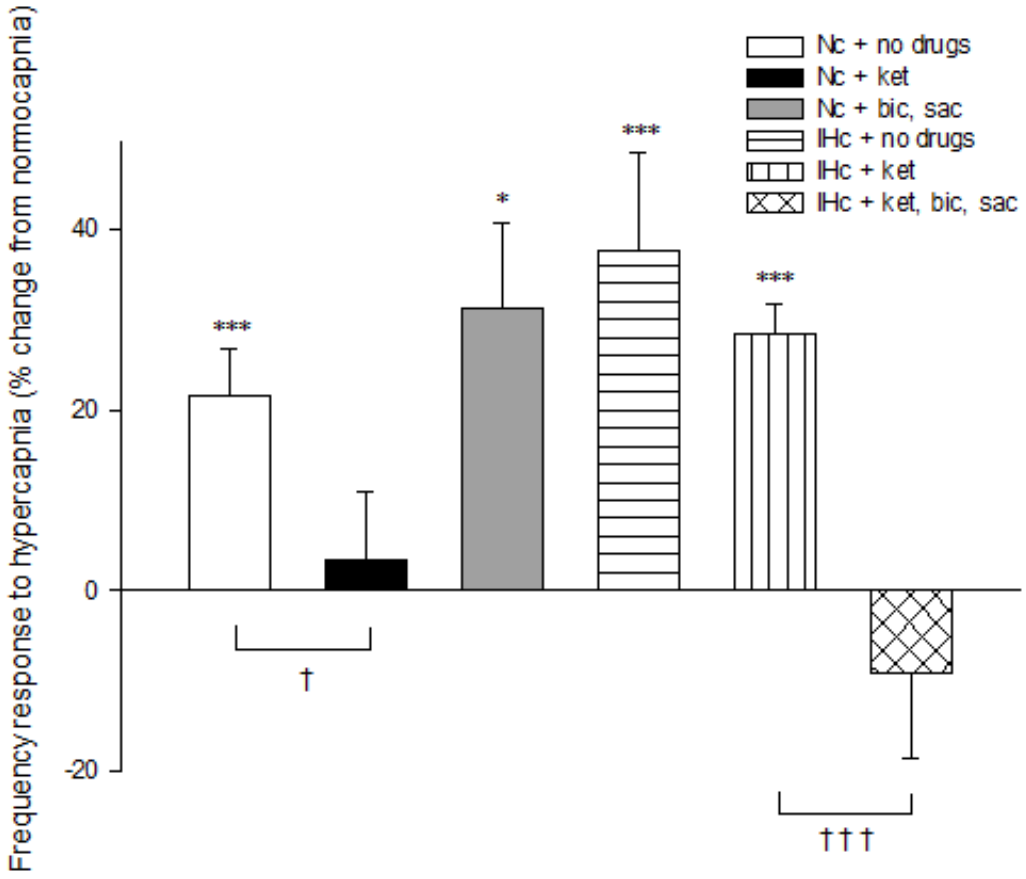

Figure 2.2 **Influence of IHc on frequency response to hypercapnia.** Hypercapnia increased ventilatory frequency, normalized to normocapnia, in preparations derived from Nc-pretreated pups without drugs (no fill bar, *n* = 20), those that were Nc-pretreated and received bicuculline (bic) and saclofen (sac, grey bar, *n* = 4), those that were IHc-pretreated and received no drugs (horizontal stripe bar, *n* = 16) and those that were IHc-pretreated and received ketanserin (ket, vertical stripe bar, *n* = 27). Each bar represents mean ± SE. Significant increase from normocapnia to hypercapnia (one-way RM-ANOVA):

*$P < 0.05$, ***$P < 0.001$. Between groups comparison of difference in response to hyper-capnia determined using one-way ANOVA: †$P < 0.05$, †††$P < 0.001$.

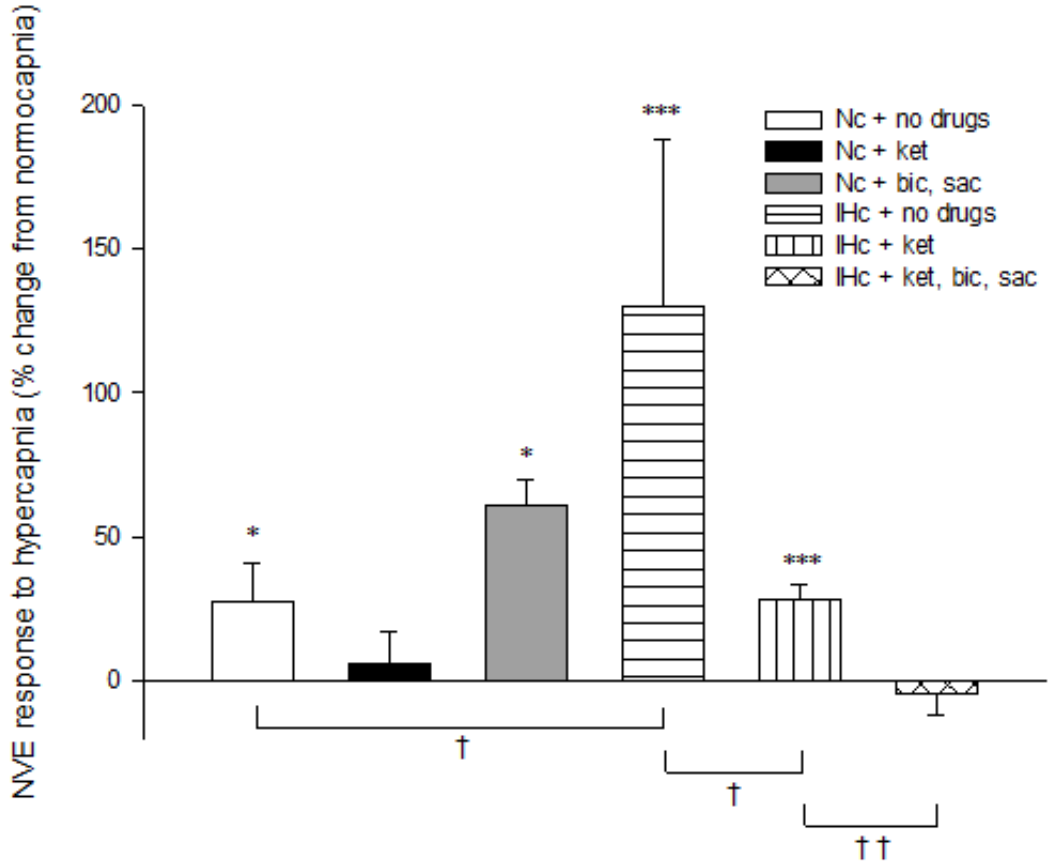

Figure 2.3 **Influence of IHc on NVE response to hypercapnia.** Hypercapnia increased ventilatory NVE, normalized to normocapnia, in preparations derived from pups that were Nc-pretreated and did not receive drugs (no fill bar), those that were Nc-pretreated and received bicuculline (bic) and saclofen (sac, grey bar), those that were IHc-pretreated and did not received drugs (horizontal stripe bar) and those that were IHc-pretreated and received ket (vertical stripe bar). Each bar represents mean ± SE. Significant increase from normocapnia to hypercapnia (one-way RM-ANOVA): *$P < 0.05$, ***$P < 0.001$. Between groups comparison of difference in response to hypercapnia determined using one-way ANOVA: †$P \leq 0.05$, ††$P < 0.01$.

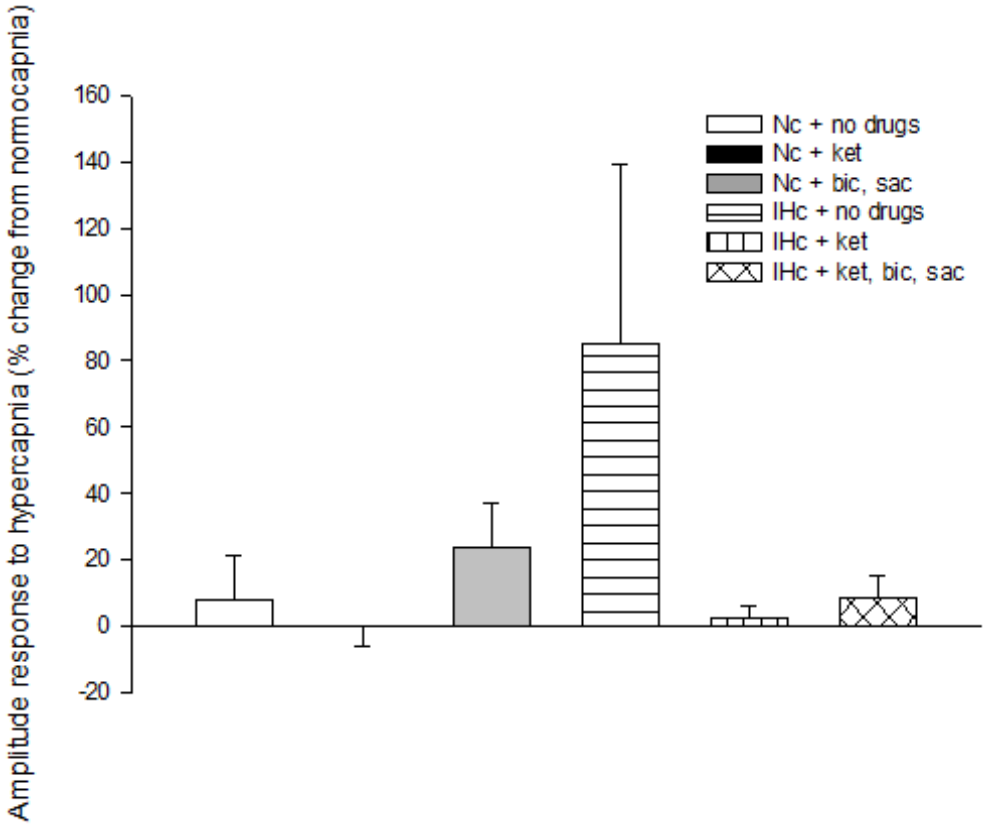

Figure 2.4 **Influence of IHc on amplitude response to hypercapnia.** Hypercapnia failed to increase phrenic burst amplitude. Each bar represents mean ± SE. There was no resolvable difference between groups, determined using one-way ANOVA.

Chapter 3

Intermittent hypercapnia enhances CO_2 responsiveness and overcomes dysfunction induced by dietary tryptophan deficiency [1]

3.1 Abstract

Serotonin (5-HT) dysfunction is thought to enhance vulnerability to the Sudden Infant Death Syndrome (SIDS) by compromising critical homeostatic reflexes. Rat dams fed a tryptophan (Trp)-deficient diet produced pups with low brainstem tissue 5-HT and exhibited a reduced ventilatory response to inspired CO_2 *in vivo* (Penatti et al., 2011). We used an *in situ* perfused brainstem preparation derived from pups of dams fed either a control or Trp-deficient diet to test the hypothesis that developmental Trp deficiency alters central CO_2/pH chemoresponsiveness. We also investigated the efficacy of a previously described intermittent hypercapnia (IHc) protocol (Mosher et al., in review) to reverse the dampened hypercapnic response induced by the dietary Trp restriction. Results indicate that the response to hypercapnia is decreased in preparations derived from animals maintained on the Trp-deficient diet. Additionally, for animals maintained on the Trp-deficient diet that were also pretreated with IHc, the reduced hypercapnic response was restored to levels comparable to control animals. We previously showed that IHc-pretreatment enhances the response to hypercapnia (Mosher et al., in review). These data further illustrate that dietary Trp restriction, likely resulting in decreased brainstem 5-HT, induces a

[1] Mosher BP, Taylor BE, Harris MB. Intermittent hypercapnia enhances CO_2 responsiveness and overcomes dysfunction induced by dietary tryptophan deficiency. Prepared for publication in *J. Appl. Physiol.*

measurable reduction of the hypercapnic ventilatory response, and that IHc-pretreatment of Trp-deficient pups restores CO_2 responsiveness to that of control animals. Results may shed light on a possible intervention that could be used to enhance the central response to hypercapnia under conditions in which it is impaired.

3.2 Introduction

The present study was conducted to provide insight into respiratory physiology and pathologies thought to result from chemosensory dysfunction, such as the Sudden Infant Death Syndrome (SIDS). SIDS, the leading cause of infant mortality between the ages of 1 month and 1 year, is defined as the sudden death of an infant that is unexplained after reviewing clinical history and cannot be explained after a thorough death scene investigation and post-mortem examination (Moon et al., 2007; Willinger et al., 1991). A considerable amount of research has investigated the causes of SIDS, but little research has been conducted to identify a potential therapy that may decrease an individuals' vulnerability to SIDS. Malnutrition during pregnancy and early postnatal life has well-documented deleterious effects on brain differentiation and growth (Gressens et al., 1997; Guesry, 1998). Much less understood are the effects of malnutrition on brainstem homeostasis.

A complete understanding of SIDS etiology remains elusive. One hypothesis is that abnormal brainstem serotonin (5-HT) mechanisms in regions responsible for homeostatic control of pH may enhance SIDS vulnerability (Duncan et al., 2010; Hodges and Richerson,

2010; Kinney et al., 2009; Paterson et al., 2006). Such vulnerability may result from dysfunction in reflex responses to potentially life threatening conditions, such as exposure to harmful CO_2 levels. This hypothesis is supported by animal studies demonstrating the physiological consequences of various levels of experimentally-induced 5-HT dysfunction (Hodges and Richerson, 2008; Hodges et al., 2008, 2011). Furthermore, post-mortem examination of infants who succumbed to SIDS show evidence of altered 5-HT mechanisms (Duncan et al., 2010; Kinney et al., 2009). Since that observation, research has indicated that the consequences of 5-HT dysfunction are widely varied, and implicates some such dysfunction in SIDS (Cummings et al., 2009; Kinney et al., 2009). One potential 5-HT-mediated process that is thought to be impaired in SIDS is CO_2 ventilatory chemoresponsiveness (Richerson, 2004).

Central CO_2 chemoresponsiveness is a complex system function that involves a limited but varied group of neuron types, brainstem sites and multiple neurotransmitter mechanisms (Feldman et al., 2003; Mitchell et al, 1990; Nattie, 2009; Nattie and Li, 2009; Putnam et al., 2004). This organization affords the potential for considerable plasticity in central CO_2 chemoresponsiveness to maintain pH homeostatic regulatory function despite dysfunctional mechanisms, injury or disease (Feldman et al., 2003). Thus, if one reflex mechanism is dysfunctional another alternative mechanism may be present or enhanced to allow an appropriate response. In addition, ventilatory responsiveness to CO_2 is a system function for which more than one single mechanism is responsible. Because of this, different mechanisms likely play larger or smaller roles during specific situations.

In addition, some mechanisms may be important enough that their dysfunction causes severe consequences. There is strong evidence for an important role of 5-HT in CO_2 chemoresponsiveness. However, certain types of 5-HT dysfunction, and even complete absence of 5-HT, does not abolish chemoresponsiveness. Recently, a study using a maternal dietary tryptophan (Trp) restriction model showed reduced medullary 5-HT and a resulting impairment of the hypercapnic ventilatory response (Penatti et al., 2011). In addition, several genetic methods have been used to investigate 5-HT and its role in CO_2 chemoresponsiveness. CO_2 responsiveness is impaired in male, but not female, *Pet-1* knockout mice, which lack ≈ 70 % of 5-HT neurons (Hodges et al., 2011). Furthermore, neonatal mice with genetic deletion of *Lmx1b* in neurons expressing *Pet-1* (*Lmx1b*$^{f/f/p}$), which have a selective and severe deficit (> 99 %) of 5-HT neurons, display a ≈ 50 % retention in the hypercapnic ventilatory response (Hodges and Richerson, 2008; Hodges et al., 2008). These examples of different types of induced 5-HT brainstem reductions demonstrate the remarkable plasticity of the ventilatory CO_2 chemoresponsive reflex to compensate for a decrease in 5-HT. The presence of any residual hypercapnic ventilatory responsiveness in *Lmx1b*$^{f/f/p}$ mice is somewhat surprising considering the importance of 5-HT. However, the organism likely accommodated for the complete loss of 5-HT through the strengthening of alternative reflex pathways. This accommodation may also be responsible for the residual hypercapnic responsiveness observed in the other examples of 5-HT reduction previously mentioned. These results suggest that the system function includes multiple mechanisms and is plastic.

In the current study, we propose that the abnormal CO_2 chemoresponsive reflex associated with dietary Trp restriction (presumably resulting in 5-HT reduction) may be reduced or reversed by stimulating reflex plasticity. Prior work has shown that CO_2 not only activates excitatory 5-HT neurons in the medullary raphé, which are known to participate in respiratory control, but also deactivates inhibitory γ-aminobutyric acid (GABA) raphé neurons (Corcoran et al., 2009; Iceman et al., 2010; Wang et al., 1998; Zhang et al., 2003). In one model, CO_2-mediated deactivation of the inhibitory GABA raphé pathway may lead to disinhibition of downstream targets, potentially stimulating breathing. Thus, in this model, the excitatory 5-HT and inhibitory GABA pathways are thought to behave in a push-pull manner to modulate ventilatory activity (Corcoran et al., 2008; Richerson, 1995). The present study used a previously described intermittent hypercapnia-pretreatment protocol (IHc; elevated arterial CO_2 concentration; Mosher et al., in review) in an attempt to reduce or overcome the abnormal ventilatory CO_2 reflexes induced by maternal dietary Trp restriction. Because our dietary restriction protocol was identical to that of Penatti et al. (2011), the abnormal ventilatory CO_2 reflexes resulting from Trp dietary restriction are thought to be due to a partial 5-HT reduction, as theirs was. This dietary restriction protocol was selected for its biological relevance and because of the link between poverty and increased SIDS risk (Bavis, 2011; Malloy and Eschbach, 2007). We tested the hypothesis that IHc-pretreatment during postnatal development enhances hypercapnic responsiveness to overcome ventilatory abnormalities induced through mater-

nal dietary Trp restriction. If induced reflex plasticity is able to overcome or reverse ventilatory CO_2 chemoresponsiveness dysfunctions that are thought to underlie SIDS, the factors that induce plasticity may be therapeutic in decreasing vulnerability to SIDS.

3.3 Methods

Experimental groups: All experiments were done in accordance with the guidelines of the "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health and were approved by the University of Alaska Fairbanks (UAF) Institutional Animal Care and Use Committees. As previously described by Penatti et al. (2011), Sprague-Dawley naive adult female rats (Simonson Laboratories) were fed normal rat chow in which tryptophan (Trp) was present in normal or reduced levels (Harlan Teklad). The control diet was composed (g/kg) of the following ingredients: 3.5 L-alanine, 12.1 L-arginine HCl, 6.0 L-asparagine, 3.5 L aspartic acid, 3.5 L-cystine, 40.0 L-glutamic acid, 23.3 glycine, 4.5 L-histidine HCl, monohydrate, 8.2 L-isoleucine, 11.1 L-leucine, 18.0 L-lysine HCl, 8.2 L-methionine, 7.5 L-phenylalanine, 3.5 L-proline, 3.5 L-serine, 8.2 L-threonine, 1.8 L-tryptophan, 5.0 L-tyrosine, 8.2 L-valine, 351.68 sucrose, 150.0 corn starch, 150.0 maltodextrin, 80.0 soybean oil, 30.0 cellulose, 35.0 mineral mix, 8.2 calcium phosphate, monobasic, monohydrate, 13.0 vitamin mix, 2.5 choline bitartrate, and 0.02 tertiary butylhydroquinone, an antioxidant. The control and Trp-deficient diets were equivalent except for the deficient diet contained a reduced 1 g/kg level of Trp, about 55 % of the estimated nutritional requirement.

Five naïve female rats received normal rat chow and water *ad libitum*, and they were bred with 5 different males. Six naïve female rats received Trp-deficient rat chow and water *ad libitum*, and they were bred with 6 different males. All animals received their respective diet *ad libitum* for 2 weeks prior to mating, during gestation and postnatally. Resulting pups also received their respective diet (Trp-deficient or control) and water *ad libitum* and were housed and maintained in the UAF Animal Care Facility on a 12 h light/dark cycle, at a room temperature of 21 °C. A total of 74 animals from both sexes were studied.

Gas pretreatments: Rat pups were exposed to intermittent hypercapnia (IHc; 8 consecutive cycles of 5 min 5 % CO_2: balance air, followed by 10 min air) or constant normocapnia as a control (Nc; Type 1-Grade D air only, as a treatment sham) each day for 5 consecutive days beginning at post-natal day 12 (P12). Entire litters were randomly assigned to either IHc- or Nc-pretreatment. Because related litter-mates, rather than randomly assigned individuals, were used as experimental subjects, multiple litters (2 to 5) received each combination of gas and pharmacological treatments. During gas treatments, dams were separated from pups and the home cage was transported to a procedure room adjacent to the animal holding area. Cages were fitted with an airtight lid with a gas inflow and outflow. In this manner, litters (ranging from 3 to 15 pups) were exposed to room temperature inlet gas, supplied at 10 l/min through a countercurrent heat exchanger. The gas outlet was connected to 1 m of 5.5 mm internal diameter tubing to pre-

vent room air infiltration without creating substantial positive pressure within the chamber. For intermittent gas exposure, a programmable digital timer and solenoid valve automatically switched inlet gases between the two sources. This apparatus produced a 10-min period of normocapnia followed by 8 repeated cycles of 5-min hypercapnia (or normocapnia in controls) and 10-min normocapnia. Valve cycling was monitored using a computerized data acquisition system (LabChart 7, ADInstruments). Pilot studies indicated that CO_2 levels in the enclosure equilibrated with inlet gas concentrations within 90 s. Chamber gas was not monitored during these treatments. After this exposure protocol, cages were removed from the enclosure, dams were returned to pups in the home cage and cages were returned to the adjacent housing facility. In no cases did pup abandonment occur following these brief maternal separations, and patterns and durations of maternal separation were equal between IHc and Nc treatment groups. Exposure protocols were repeated on 5 consecutive days, at approximately the same time each day.

In situ assessment of CO_2 chemoresponsiveness: At least 7 days following IHc- or Nc-pretreatment, hypercapnic ventilatory responsiveness was assessed using the *in situ* arterially perfused brainstem preparation as previously described (Corcoran et al., 2013; Toppin et al., 2007, after Paton, 1996). Briefly, animals were anesthetized with isoflurane (5 %, vaporized in 100 % O_2) and pretreated with heparin sodium (500 units, I.P.). Animals were bisected subdiaphragmatically and submerged in an ice-chilled artificial cerebral spinal fluid (aCSF) containing (mM in H_2O): $MgSO_4 \cdot 7H_2O$, 1; $NaH_2PO_4H_2O$, 1.25; KCl, 4; $NaHCO_3$, 24; NaCl, 115; D-glucose, 10; $CaCl_2 \cdot 2H_2O$, 2). The forebrain rostral to the colliculi

was removed by aspiration, and fur, skin and viscera were removed. The diaphragm was separated from the body wall with care taken to ensure integrity of the phrenic nerve. The preparation was moved to the recording station where the descending aorta was cannulated using a double-lumen catheter (Ø 1.25 mm, Braintree Scientific) and perfused retrogradely from a reservoir containing 350 ml aCSF (with 13 g/l ficoll 70, Sigma, added as an osmotic agent). The perfusate was first equilibrated with 95 % O_2-5 % CO_2 (normocapnia, pH 7.4, P_{CO_2} of 33 mmHg). Equilibration mixtures were produced from O_2 and CO_2 using a precision gas mixer (GSM3, CWE) and verified with a CO_2 analyzer (CD-3A, Applied Electrochemistry). Normocapnic (baseline) conditions approximated normocapnic plasma *in vivo*. Lacking hemoglobin, solution hyperoxia ($Po_2 \approx 600$ mmHg) was necessary to maintain O_2 content sufficient to meet tissue metabolic demands. This unavoidable hyperoxia was constant under all conditions. Perfusate was warmed to 32 °C, and circulated through a bubble trap and particle filters (25 µm, 45 µm; Millipore) prior to entering the aorta. Perfusate passing through the animal was collected and recycled to the reservoir. The neuromuscular blocker gallamine triethiodide (20 mg/l), and the vaso-constriction hormone vasopressin (5 µM) were added to the perfusate. The aortic perfu-sion pressure was adjusted to 70-80 mmHg using an adjustable perfusate bypass valve and a bolus of sodium cyanide (50 µl 0.1 % solution) was injected into the perfusate line to transiently stimulate peripheral chemoreceptors (Dutschmann et al., 2000). After par-tial pulmonectomy, the phrenic nerve was exposed and aspirated into a glass capillary suction electrode pulled to a diameter that ensured adequate seal on the nerve.

The signal was amplified (×10,000; DAM50, WPI) and filtered (band-pass 300 Hz – 1 kHz). Using a computerized data acquisition system (Powerlab, ADInstruments), data were digitized at 1 k Samples/s and digitally integrated through full wave rectification and 50 ms moving average on a duplicate channel. Phrenic burst frequency (f, bursts/min) was determined by counting the number of phrenic bursts occurring during 60 s of normocapnia immediately preceding the gas challenge, the final 60 s of hypercapnia, and 60 s during normocapnic recovery 5 min after a return to baseline. Phrenic burst amplitude was derived from the mean integrated peak height of all bursts within these 60 s periods, expressed in arbitrary units within each preparation and as a proportional change within a preparation in response to the gas challenge. Neural minute ventilation (NVE) was calculated as the product of burst frequency and amplitude, again expressed in arbitrary units and as a proportional change with treatment (Eldridge, 1971).

Gas Challenges: Preparations were maintained on normocapnic perfusate for at least 1 h. For the gas challenge, gas equilibrating the perfusate was switched in sequence to 5-min periods of hypocapnia (96.5 % O_2-3.5 % CO_2; pH 7.5; P_{CO_2} ≈ 23 mmHg) followed by 5-min periods of hypercapnia (91 % O_2-9 % CO_2; pH 7.2; P_{CO_2} ≈ 60 mmHg) and subsequently returned to baseline normocapnia. The hypercapnic challenge conditions approximated those occurring during a 4 % increase in inspired CO_2.

Data and statistical analyses: Ventilatory parameters were quantified from the integrated neurograms. Frequency (f; the number of bursts per unit time), amplitude (the mean integrated peak amplitude of bursts), and a neural correlate of minute ventilation

(NVE; frequency•amplitude) were calculated from periods of normocapnia or hypercapnia. Hypercapnic responsiveness was determined from ventilatory parameters recorded during baseline normocapnia and hypercapnia. This was quantified by deriving a relative hypercapnic response, calculated by expressing the difference in parameter values between normocapnia and hypercapnia, normalized as a percentage relative to values occurring during normocapnia prior to gas challenge (responsiveness = [value during hypercapnia – value during normocapnia] / value during normocapnia).

Data Parsing: We and others have demonstrated that the *in situ* rat brainstem preparation exhibits clear responsiveness to hypercapnia and provides an appropriate model for the study of mechanisms contributing to chemosensitivity (Corcoran et al., 2013; Day and Wilson, 2007; Iceman et al., 2013; Toppin et al., 2007). These prior studies have illustrated that the response is most clearly manifested by changes in burst frequency and the index of minute ventilation, NVE. Our pilot investigations suggested the possibility that preparations exhibiting a relatively high initial burst frequency have a lower apparent responsiveness to hypercapnia, which suggests a frequency limitation. The present study was designed to determine chemosensory mechanisms, necessitating a focus on responsive preparations free of potential confounding factors.

To remove the potential confounding influence of dampened chemoresponsiveness in preparations with elevated baseline burst frequencies, we subjected the data to the following parsing protocol. In preparations exposed to neither dietary restriction nor IHc, we plotted the relationship between hypercapnic responsiveness and initial burst

frequency and determined a linear regression ($P < 0.001$; $R^2 = 0.74$) described by the equation hypercapnic responsiveness (HcR) = 192 – 3.45 (initial frequency). The highest sensitivity observed in any of these preparations was 184.6. A half-maximum value was used to determine a threshold initial burst frequency identifying preparations for which high initial frequency could confound subsequent responsiveness. Preparations having an initial burst frequency above this threshold were not included in the dataset. When so parsed, 16 of 74 preparations (21 %) were removed from analyses; a total of 58 preparations contributed to the data set.

One-way repeated-measures analysis of variance (RM-ANOVA) was used to compare frequency, amplitude or NVE parameters before and during hypercapnia for each preparation, which quantified hypercapnic responsiveness for these three ventilatory parameters. One-way ANOVA was used to compare the effect of dietary restriction and IHc treatment on the hypercapnia-induced change in each parameter. For each parameter (f, amplitude and NVE) in each experiment we calculated the % change from normocapnia = [(parameter during hypercapnia – parameter during normocapnia) / parameter during normocapnia)] • 100 %. Values reported in the text are mean ± standard error. To compare the mean hypercapnic responsiveness for each parameter between groups, a Tukey post-hoc analysis was used following a significant ANOVA.

3.4 Results

Burst discharge recordings: All our recorded phrenic neurograms displayed a "eupneic" pattern (Fig. 3.1) characteristic of this preparation (Eldridge, 1971; St.-John and Paton, 2000), and displayed this pattern throughout normocapnia, hypercapnia and normocapnic recovery.

Preparations derived from pups maintained on the control diet that were Nc-pretreated had an average normocapnic burst frequency of 20.7 ± 1.26 bursts/min (data from Chapter 2). Hypercapnic responsiveness in these preparations was also typical of past reports (Corcoran et al., 2008; Mosher et al., in review; St.-John et al., 2007), reflected by an increase in burst frequency and amplitude, and thus NVE as well, during hypercapnia compared to normocapnia (Fig. 3.1).

Hypercapnic responsiveness with control and tryptophan-deficient diets: Hypercapnic responsiveness is characterized by proportional change from normocapnic values, expressed as a % change (Figs. 3.2, 3.3, 3.4). In preparations derived from pups exposed to the control diet and Nc-pretreatment, normalized hypercapnic burst frequency values were elevated (22 ± 5.0 %) from baseline values indicating hypercapnic responsiveness ($P < 0.001$; Fig. 3.2, data reported in Chapter 2). Similarly, CO_2 responsiveness was also shown by a 27 ± 14 % increase in NVE with hypercapnia in these preparations ($P < 0.05$; Fig. 3.3, data reported in Chapter 2). In preparations from pups maintained on the Trp-deficient diet that were Nc-pretreated, burst frequency also increased 27.1 ± 9.08 % with hypercapnia ($P < 0.001$; Fig. 3.2), although NVE was unchanged (Fig. 3.3).

Effect of IHc on hypercapnic responsiveness with control and tryptophan-deficient diets: Preparations from pups maintained on the control diet that were IHc-pretreated increased burst frequency and NVE with hypercapnia (38 ± 11 %, 130 ± 58 % respectively; $P < 0.001$; Figs. 3.2, 3.3). For preparations from pups maintained on the control diet that were IHc-pretreated, hypercapnic responsiveness in burst frequency was equal while increases in NVE were greater than those observed in the Nc-pretreated group ($P < 0.05$; Fig. 3.3). Hypercapnia increased burst frequency in preparations from pups maintained on the Trp-deficient diet that were IHc-pretreated (16 ± 3.1 %; $P < 0.001$; Fig. 3.2; equal to control diet Nc-pretreated controls), and NVE also increased by proportions equal to those of control diet Nc-pretreated controls (29 ± 7.0 %; $P < 0.01$; Fig. 3.3; equal to control diet Nc-pretreated controls).

3.5 Discussion

Our study assessed if pretreating with IHc during postnatal development could induce ventilatory plasticity to overcome the reduced CO_2 chemoresponse associated with dietary Trp restriction. Penatti et al. (2011) demonstrated that rats maintained on the same dietary Trp restriction protocol used in the current study had a 41–56 % reduction in brainstem medullary 5-HT. This model has been suggested to be particularly pertinent to studies of infant vulnerability to SIDS as dietary-restricted pups exhibit a partial tissue 5-HT reduction and represent a close approximation to the 5-HT defect in SIDS infants (Penatti et al., 2011). Although we are not certain, we assume that a similar reduction of

medullary 5-HT was induced by the Trp-deficient diet in the current study. In addition, similar CO_2 ventilatory chemoresponsive deficits were observed.

Our results confirm that the rat *in situ* perfused brainstem preparation does exhibit CO_2 responsiveness (Corcoran et al., 2013; Day and Wilson, 2007). Preparations derived from animals that were maintained on the Trp-deficient diet and Nc-pretreated did not exhibit NVE CO_2 chemoresponsiveness. Although this group did exhibit ventilatory frequency CO_2 chemoresponsiveness, this was not particularly surprising as the dietary Trp restriction previously resulted in only a modest dysfunction in CO_2 chemoresponsiveness (Penatti et al., 2011).

The hypercapnic ventilatory response is a complex system function that involves multiple neuron types, brainstem sites and neurotransmitter mechanisms (Feldman et al., 2003; Nattie, 2009; Nattie and Li, 2009; Putnam et al., 2004). Diverse mechanisms contribute to this system function, allowing for plasticity to provide homeostatic regulation despite dysfunction in any one mechanism (Feldman et al., 2003).

We aimed to evaluate if plasticity induced through IHc-pretreatment could overcome the impaired CO_2 chemoresponse associated with dietary Trp restriction. We propose that CO_2 chemoresponsive plasticity can be induced by IHc-pretreatment to precondition the system and enhance alternate mechanisms to compensate for the dysfunctional processes induced by Trp restriction.

We used the same IHc-pretreatment regimen that we previously described in the first report of an IHc-induced enhancement of CO_2 chemoresponsiveness (Mosher et al.,

in review). In these experiments, IHc-pretreatment was initiated on P12 and continued daily through P16. Protocols were designed to administer cycles of mild hypercapnia to pups, without altering maternal conditions and while limiting maternal separation. This developmental period was chosen to follow the apparent critical period proximal to P12 where rat CO_2 chemosensitivity is altered (Putnam et al., 2005; Wong-Riley and Liu, 2005; 2008), and the developmental period beyond which CO_2 chemosensitive 5-HT neurons have been identified *in vitro* (Wang and Richerson, 1999). Prior attempts to influence hypercapnic responsiveness showed no influence when a comparable IHc protocol was conducted between P7 and P14 (Steggerda et al., 2009). It is likely that the developmental timing and/or nature of the IHc stimulus influences resultant plasticity.

We show that the plasticity induced by IHc-pretreatment is capable of overcoming the CO_2 chemoresponse dysfunction produced by dietary Trp restriction. Animals maintained on the Trp-deficient diet did not exhibit NVE CO_2 chemoresponsiveness in preparations that were Nc-pretreated. Both frequency and NVE CO_2 chemoresponsiveness persisted, however, in preparations maintained on the Trp-deficient diet that were IHc-pretreated. The frequency and NVE responses were no different than those observed in control diet Nc-pretreated preparations. In addition, both frequency and NVE CO_2 chemoresponsiveness for preparations maintained on the Trp-deficient diet that were IHc-pretreated were no different than those observed in Trp-deficient preparations that

were Nc-pretreated. We attribute the retention of relatively normal CO_2 chemoresponsiveness in the Trp-deficient IHc-pretreated group to plasticity imparted by IHc-pretreatment.

The organization of respiratory chemosensitivity as a system function grants the potential for considerable plasticity. Not only do particular mechanisms play differential roles in overall system sensitivity under particular conditions, the involvement of multiple mechanisms permits homeostatic pH regulation despite partial dysfunction, injury or disease (Feldman et al., 2003). Thus, if one reflex mechanism is dysfunctional, alternative mechanisms can often accommodate an appropriate response. We have previously shown that IHc-pretreatment enhances a ketanserin-insensitive mechanism not normally critical for CO_2 responsiveness. In addition we have demonstrated that plasticity induced by IHc-pretreatment is due to the enhancement of bicuculline- and saclofen-sensitive mechanisms (Mosher et al., in review). It may be that the plasticity induced by IHc-pretreatment observed in the current study is also due to similar mechanisms. Further investigations are necessary.

Critique of methods: Our use of the dietary restriction model is based on the earlier finding that this Trp-restriction induced a partial tissue 5-HT reduction that represents a physiologically relevant animal model to the partial 5-HT dysfunction associated with SIDS (Penatti et al., 2011). We confirm the results of this earlier study both in that the dietary Trp restriction induced a measurable deficit in CO_2 chemoresponsiveness and that this deficiency is subtle. However, despite the similarity in the dysfunctional CO_2

chemoresponse, we are not able to assume that the dysfunction that we observe is due to 5-HT reduction as seen by Penatti et al., (2011) as we did not measure tissue 5-HT levels. In our hands, dietary restriction abolished the NVE component of CO_2 chemoresponsiveness, while in Penatti's study this dietary treatment reduced the magnitude of ventilatory CO_2 chemoresponsiveness resolved from changes in minute ventilation normalized to metabolic rate (V_E/VO_2). Also, as was acknowledged in Pennati's report (2011), our use of the dietary restriction model incorporated an unavoidable pseudoreplication. Control and Trp-deficient diets were administered to the dam 2 weeks prior to mating, during gestation and postnatally and maintained in all of the pups in a litter. Although it would be a more powerful experimental design to study only one pup from multiple treated litters, we also chose to study all pups in fewer litters to avoid excess animal use. Additionally, although animals in our study were maintained on the same control and Trp-deficient diets, there may have been unforeseen variables that could have resulted in different levels of tissue 5-HT reduction than seen by Penatti et al. (2011).

3.6 Conclusion

In this study, we used a previously described biologically relevant model for the Sudden Infant Death Syndrome susceptibility on account of poor nutrition and subsequent diminished levels of brainstem 5-HT (Penatti et al., 2011). The degree to which ventilatory CO_2 chemoresponsiveness was impacted by dietary Trp restriction was then observed. This

dietary Trp restriction model was used to test the effectiveness of a potential therapy, IHc-pretreatment, which was designed to induce reflex plasticity.

We confirm the finding that animals maintained on a Trp-deficient diet exhibit a partially dysfunctional hypercapnic response, suggesting contributions mediated by dietary Trp are important for CO_2 responsiveness under these conditions. We show that IHc-pretreatment induces plasticity such that CO_2 chemoresponsiveness is maintained despite dietary Trp restriction.

Serotonergic dysfunction likely contributes to a number of pathologies, including SIDS, by compromising homeostatic reflexes such as hypercapnic chemoresponsiveness. It may be that plasticity induced by IHc-pretreatment could enhance pH homeostatic reflex efficacy and potentially reduce vulnerabilities to conditions such as SIDS and other respiratory pathologies thought to be the result of CO_2 chemoresponse dysfunction. A number of critical factors remain unknown. These include exact mechanisms involved in plasticity induced by IHc-pretreatment, the longevity of this plasticity, and potential developmental sensitivities to plasticity induced by IHc-pretreatment. The clear potential to induce CO_2 chemoresponse plasticity, however, provides possible targets for therapeutic intervention to reverse CO_2 chemoresponsive dysfunction.

3.7 References

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3.8 Figures

See below where figures are displayed on full pages.

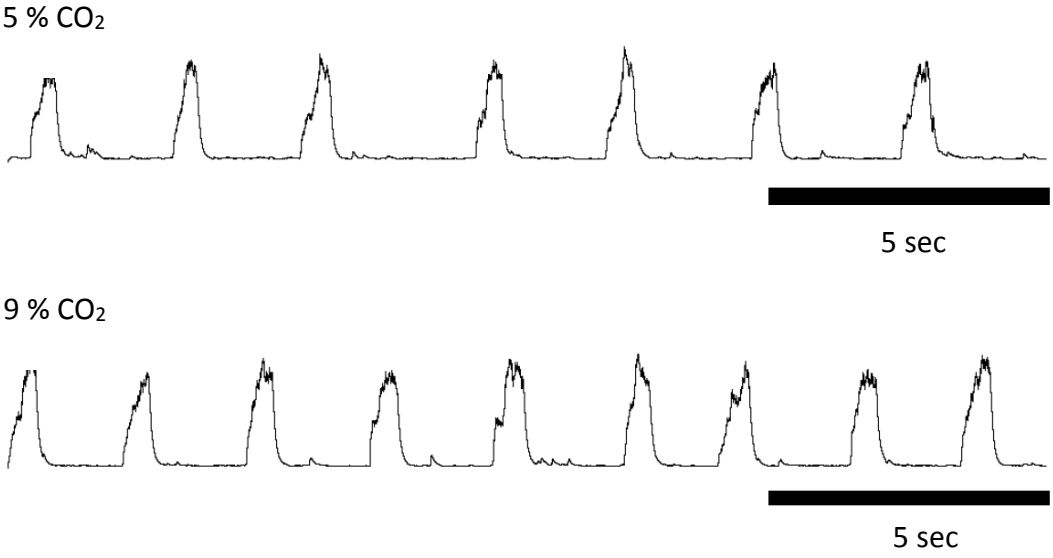

Figure 3.1 **Eupneic phrenic ventilatory burst *in situ.*** Typical phrenic neurogram recording with spikes indicating respiratory bursts. During the *in situ* preparation when the organism was exposed to perfusate containing 9 % CO_2 (hypercapnia; bottom panel) there was an increase in both frequency and NVE (frequency*amplitude) of respiratory events, when compared to experiencing 5 % CO_2 (normocapnia; top panel). Both of the above panels are representative tracings from the last minute of the indicated CO_2 treatment. Tracings taken from a preparation derived from a pup that received control diet and Nc-pretreatment without pharmacological manipulation. Tracings reported here are the same used in Chapter 2.

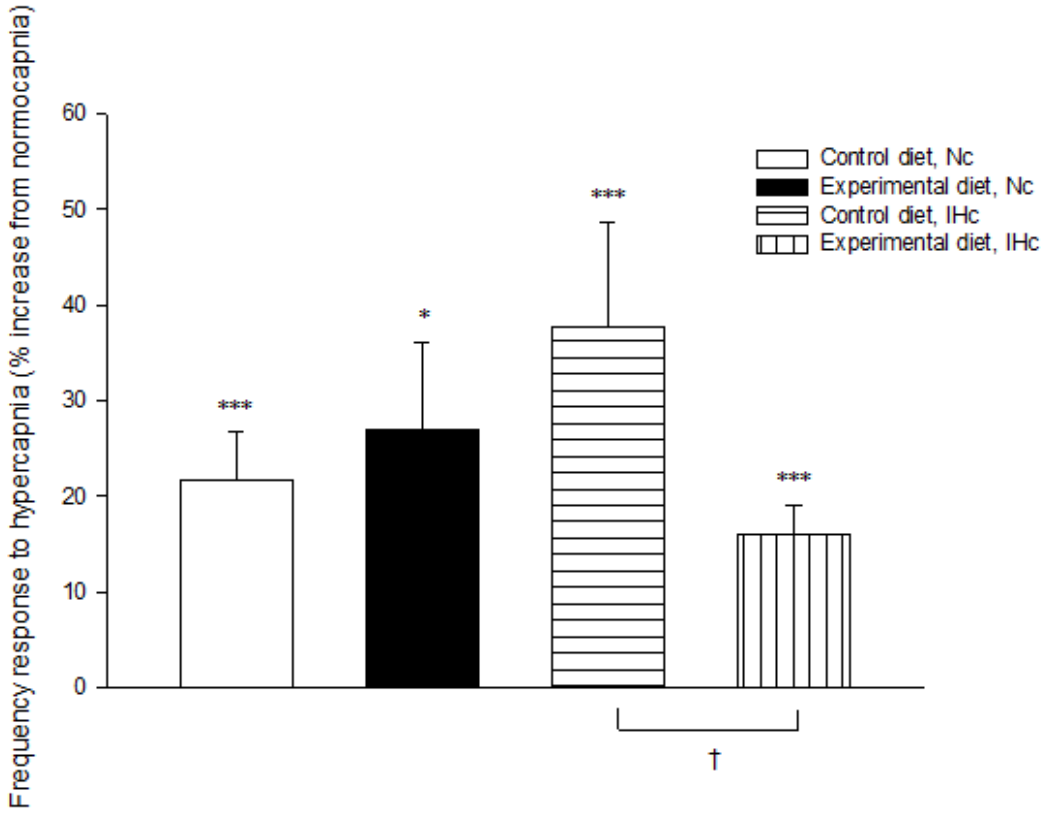

Figure 3.2 **Influence of IHc on frequency response to hypercapnia for preparations derived from animals maintained on control and tryptophan-deficient diets.** Hypercapnia increased ventilatory f, normalized to normocapnia, in preparations derived from pups that received control diet and were Nc-pretreated (no fill bar, *n* = 20, same data as reported in Chapter 2), those that received experimental diet and were Nc-pretreated (solid fill bar, *n* = 9), those that received control diet and IHc-pretreatment (horizontal stripe bar, *n* = 16) and those that received experimental diet and IHc-pretreatment (vertical stripe bar, *n* = 13). Each bar represents mean ± SE. Significant increase from normocapnia to hypercapnia (one-way RM-ANOVA): **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Between groups comparison of difference in hypercapnic responsiveness determined using one-way ANOVA: †$P < 0.05$.

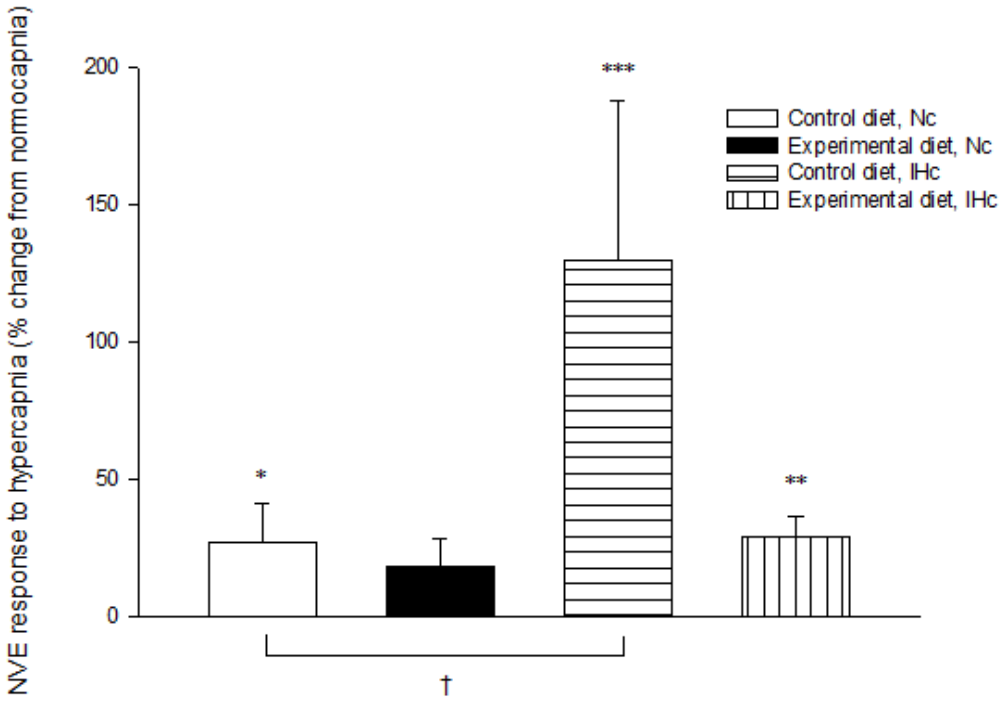

Figure 3.3 **Influence of IHc on NVE response to hypercapnia for preparations derived from animals maintained on control and tryptophan-deficient diets.** Hypercapnia increased ventilatory NVE, normalized to normocapnia, in preparations derived from pups that received control diet and were Nc-pretreated (no fill bar, *n* = 20, same data as reported in Chapter 2), those that received control diet and were IHc-pretreated (horizontal stripe bar, *n* = 16) and those that received experimental diet and IHc-pretreatment (vertical stripe bar, *n* = 13). Each bar represents mean ± SE. Significant increase from normocapnia to hypercapnia (one-way RM-ANOVA): **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Between groups comparison of difference in hypercapnic responsiveness determined using one-way ANOVA: †*P* < 0.05.

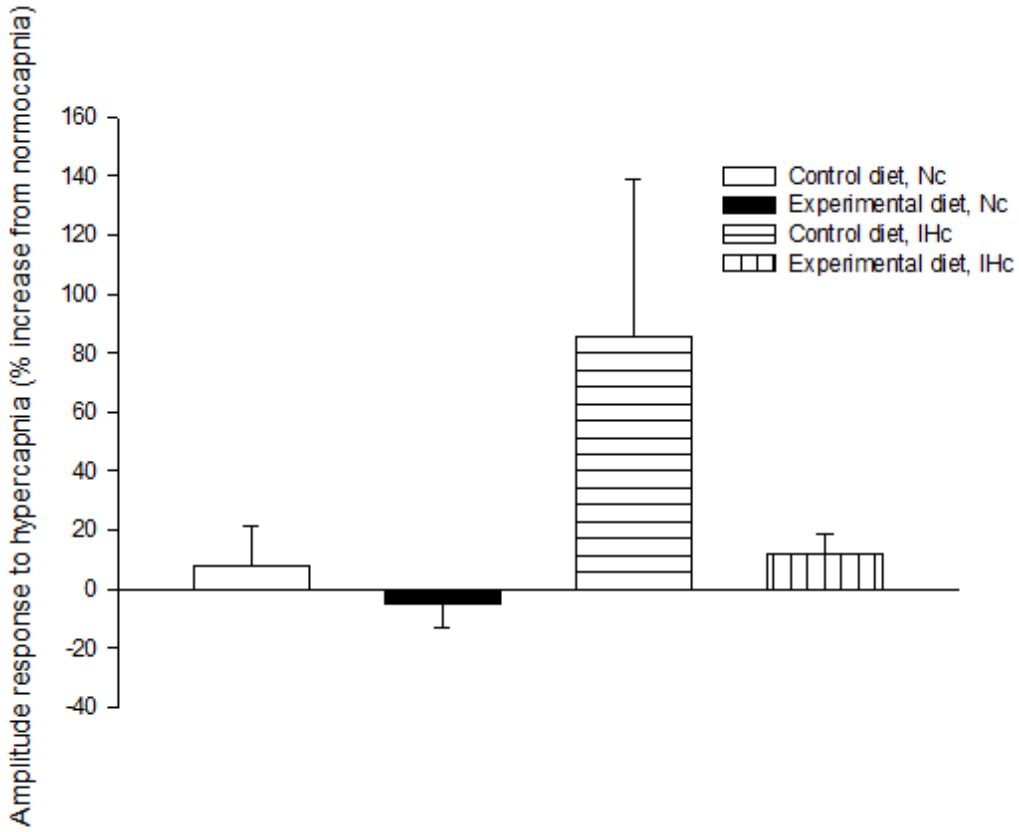

Figure 3.4 **Influence of IHc on amplitude response to hypercapnia for preparations derived from animals maintained on control and tryptophan-deficient diets.** Hypercapnia failed to increase ventilatory amplitude, normalized to normocapnia. Each bar represents mean ± SE. Between groups comparison of difference in hypercapnic responsiveness determined using one-way ANOVA failed to reveal any difference between groups. Bar describing preparations without drugs that were Nc-pretreated (no fill bar) are data previously reported in Chapter 2.

Chapter 4

Intermittent hypercapnia induces long-lasting plasticity to enhance ventilatory CO_2 responsiveness to overcome ketanserin-sensitive dysfunction[1]

4.1 Abstract

Abnormal homeostatic responses to increased levels of CO_2 are associated with numerous dangerous or life-threatening disorders. We test the hypothesis that exposure to mild intermittent hypercapnia (IHc) during postnatal development will induce long-lasting respiratory plasticity, due to the strengthening of non ketanserin-sensitive mechanisms. Rat pups were exposed to a previously described IHc protocol (Mosher et al., in review) each day for 5 days beginning at postnatal day 12 (P12), and were subsequently assessed for CO_2/pH chemoresponsiveness using an unanesthetized juvenile rat *in situ* perfused decerebrate brainstem preparation. Neuroventilatory response to CO_2 was tested during three age groups; P21-35, P36-50 and P51-65. Such CO_2 chemoresponsiveness was greatly enhanced by IHc-pretreatment through the strengthening of non-ketanserin-sensitive contributions resulting in preserved CO_2 chemoresponsiveness when ketanserin-sensitive mechanisms were compromised. Results indicate that IHc-pretreatment induces long-lasting CO_2 chemoresponsive plasticity and strengthens non-ketanserin-sensitive contributions persisting far into adult life (at least through P65).

[1] Mosher BP, Taylor BE, Harris MB. Intermittent hypercapnia induces long-lasting plasticity to enhance ventilatory CO_2 responsiveness to overcome ketanserin-sensitive dysfunction. In preparation for publication in *Resp. Physiol. Neurobiol.*

4.2 Introduction

Enabling animals to adapt to changes in environmental conditions and behavior, plasticity is a fundamental characteristic of neural systems. A considerable amount of research in recent years has revealed that the neural mechanisms controlling respiration are capable of exhibiting remarkable plasticity. Central CO_2 chemoreception is a complex system function that involves a limited but varied group of neuron types, brainstem sites and multiple neurotransmitter mechanisms (Feldman et al., 2003; Mitchell et al., 1990; Nattie, 2009; Nattie and Li, 2009; Putnam et al., 2004). Different mechanisms contribute to CO_2 responsiveness under different conditions. Organization of CO_2 chemosensitivity as a system function affords the potential for considerable plasticity. Not only could a specific mechanism play a differential role in overall system sensitivity under specific conditions, the involvement of multiple mechanisms may allow homeostatic regulation despite partial dysfunction, injury or disease (Feldman et al., 2003). Thus, if one reflex mechanism is dysfunctional, alternative mechanisms likely facilitate an appropriate response.

Many protocols using different respiratory chemoreceptor stimuli (hypoxia and hypercapnia), durations, intensities and patterns have been shown to evoke distinct forms of plasticity in respiratory control. These resulting forms of plasticity often differ in their effect (facilitation or depression) on different ventilatory parameters (frequency and tidal volume), their time course (seconds to years) and response to different challenges (hypoxia and hypercapnia). Thus, the specific stimulus paradigm appears to be of great importance.

Hypoxia-induced respiratory plasticity is widely considered to be the most thoroughly studied and best understood form of respiratory plasticity. Depending on the specific exposure protocol, various forms of plasticity may be induced. After a single hypoxic episode, a short-term depression of phrenic motor output (post-hypoxia frequency decline) is observed in anesthetized rats (Coles and Dick, 1996). However, when intermittent hypoxia is administered, various unique forms of plasticity are elicited. Elevated respiratory activity during normoxic exposures between successive hypoxic episodes is often observed, reflecting the development of long-term facilitation (LTF; Powell et al., 1998). Persistent elevation of respiratory motor output, lasting minutes to hours, is the subsequent result of 3-10 hypoxic episodes, with each episode varying in duration depending on specific protocol (Mitchell et al., 2001). Furthermore, LTF is elicited by intermittent but not continuous hypoxia (Baker and Mitchell, 2000). If intermittent hypoxia persists, different mechanisms of plasticity are evoked. For example, chronic intermittent hypoxia augments the short-term hypoxic ventilatory response, eliminates post-hypoxia frequency decline, and enhances LTF in rats (Ling et al., 2001). Just as different hypoxic stimulus protocols vary in their capacity to evoke respiratory plasticity, hypercapnic stimulus protocols also induce respiratory plasticity to varying degrees.

Although receiving less attention, and relatively poorly understood, hypercapnia is also capable of eliciting various forms of respiratory plasticity. In contrast to intermittent hypoxia, intermittent hypercapnia (≈ 10 % inspired CO_2) has been reported to elicit long-term depression (LTD), a long-lasting decrease in the frequency and amplitude of

respiratory motor output (Bach and Mitchell, 1998). However, LTD is not evoked by less severe levels of hypercapnia (≈ 5 % CO_2). In contrast to the previous report by Bach and Mitchell (1998), Baker et al. (2001) found that intermittent hypercapnia did not elicit significant LTD of phrenic amplitude, but significant LTD of burst frequency was resolvable. In contrast to episodic hypercapnia, Baker et al. (2001) found that continuous hypercapnia did elicit prolonged LTD. Steggerda et al. (2009) examined the effects of daily exposure to intermittent hypercapnia on the ventilatory response to subsequent hypercapnic and hypoxic exposure in neonatal rat pups. In response to a subsequent hypercapnia challenge, there was no significant difference in the ventilatory response between control and intermittent hypercapnia-exposed groups. In contrast, intermittent hypercapnia-exposed rat pups exhibited an enhanced ventilatory response to a hypoxic challenge with an increase in minute diaphragmatic electromyogram (EMG; Steggerda et al., 2009). In addition, rat pups that were exposed to perinatal hypercapnia exhibited only a transient reduction in the hypercapnic ventilatory response (Bavis et al., 2006). Collectively, these data suggest that the duration, intensity and pattern of chemosensory stimuli protocol used are of great importance when investigating the respiratory plasticity evoked by both hypoxia and hypercapnia exposure protocols.

Studying respiratory plasticity may provide insights into mechanisms that control normal development of the respiratory control system and that enable flexibility throughout life when confronted with changing environmental conditions. In addition, an understanding of respiratory plasticity may yield insights into various respiratory pathologies,

thereby providing the rationale for therapeutic intervention in cases of respiratory dysfunction, such as the Sudden Infant Death Syndrome (SIDS).

We propose that the CO_2 chemoresponsive plasticity invoked by an intermittent hypercapnia (IHc) protocol, which has previously been shown to precondition the system and enhance multiple chemosensory mechanisms (Mosher et al., in review) is capable of inducing long-lasting respiratory plasticity that persists far into life. We test the hypothesis that IHc-pretreatment during postnatal development will induce long-lasting respiratory plasticity.

Abnormal homeostatic responses to increased levels of CO_2 are associated with numerous dangerous or life-threatening disorders, including SIDS. If induced reflex plasticity is sufficient to overcome or reverse ventilatory CO_2 chemoresponsiveness dysfunctions, then interventions that induce plasticity could be therapeutic in augmenting CO_2 chemoresponsiveness and decreasing vulnerability.

4.3 Methods

Experimental groups: All experiments were done in accordance with the guidelines of the "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health and were approved by the University of Alaska Fairbanks (UAF) Institutional Animal Care and Use Committees. Sixteen naïve rat dams received normal rat chow and water *ad libitum*, and they were bred with 16 males. Sprague-Dawley rats were used in all experiments (Simonson Laboratories). Resulting pups also received food and water *ad libitum* and

were housed and maintained in the UAF Animal Care Facility on a 12 h light/dark cycle. A total of 49 animals from both sexes were used in these studies.

Gas pretreatments: Rat pups were exposed to intermittent hypercapnia (IHc; 8 consecutive cycles of 5 min 5 % CO_2: balance air, followed by 10 min air) or constant normocapnia as a control (Nc; Type 1-Grade D air only, as a treatment sham) each day for 5 consecutive days beginning at post-natal day 12 (P12). Entire litters were randomly assigned to either IHc- or Nc-pretreatment. Because related litter-mates, rather than randomly assigned individuals, were used as experimental subjects, multiple litters (2 to 3) received each combination of gas and pharmacological treatments. During gas treatments, dams were separated from pups and the home cage was transported to a procedure room adjacent to the animal holding area. Cages were fitted with an airtight lid with a gas inflow and outflow. In this manner, litters (ranging from 3 to 8 pups) were exposed to room temperature inlet gas, supplied at 10 l/min through a countercurrent heat exchanger. The gas outlet was connected to 1 m of 5.5 mm internal diameter tubing to prevent room air infiltration without creating substantial positive pressure within the chamber. For intermittent gas exposure, a programmable digital timer and solenoid valve automatically switched inlet gases between the two sources. This apparatus produced a 10-min period of normocapnia followed by 8 repeated cycles of 5-min hypercapnia (or normocapnia in controls) and 10-min normocapnia. Valve cycling was monitored using a computerized data acquisition system (LabChart 7, ADInstruments). Pilot studies indicated that CO_2 levels in the enclosure equilibrated with inlet gas concentrations within 90

s. Chamber gas was not monitored during these treatments. After this exposure protocol, cages were removed from the enclosure, dams were returned to pups in the home cage and cages were returned to the adjacent housing facility. In no cases did pup abandonment occur following these brief maternal separations, and patterns and durations of maternal separation were equal between IHc- and Nc-pretreatment groups. Exposure protocols were repeated on 5 consecutive days, at approximately the same time each day.

In situ assessment of CO_2 chemoresponsiveness: At least 7 days following IHc- or Nc-pretreatments, hypercapnic ventilatory responsiveness was assessed using the *in situ* arterially perfused brainstem preparation as previously described (Corcoran et al., 2013; Toppin et al., 2007, after Paton, 1996). Briefly, animals were anesthetized with isoflurane (5 %, vaporized in 100 % O_2) and pretreated with heparin sodium (500 units, I.P.). Animals were bisected subdiaphragmatically and submerged in an ice-chilled artificial cerebral spinal fluid (aCSF) containing (mM in H_2O): $MgSO_4 \cdot 7H_2O$, 1; $NaH_2PO_4H_2O$, 1.25; KCl, 4; $NaHCO_3$, 24; NaCl, 115; D-glucose, 10; $CaCl_2 \cdot 2H_2O$, 2). The forebrain rostral to the colliculi was removed by aspiration, and fur, skin and viscera were removed. The diaphragm was separated from the body wall with care taken to ensure integrity of the phrenic nerve. The preparation was moved to the recording station where the descending aorta was cannulated using a double-lumen catheter (Ø 1.25 mm, Braintree Scientific) and perfused retrogradely from a reservoir containing 350 ml aCSF (with 13 g/l ficoll 70, Sigma, added as an osmotic agent). The perfusate was first equilibrated with 95 % O_2-5 % CO_2 (normocapnia, pH 7.4, P_{CO_2} of 33 mmHg). Equilibration mixtures were produced from O_2

and CO_2 using a precision gas mixer (GSM3, CWE) and verified with a CO_2 analyzer (CD-3A, Applied Electrochemistry). Normocapnic (baseline) conditions approximated normocapnic plasma *in vivo*. Lacking hemoglobin, solution hyperoxia (Po_2 ≈ 600 mmHg) was necessary to maintain O_2 content sufficient to meet tissue metabolic demands. This unavoidable hyperoxia was constant under all conditions. Perfusate was warmed to 32 °C, and circulated through a bubble trap and particle filters (25 µm, 45 µm; Millipore) prior to entering the aorta. Perfusate passing through the animal was collected and recycled to the reservoir. The neuromuscular blocker gallamine triethiodide (20 mg/l), and the vaso-constricting hormone vasopressin (5 µM) were added to the perfusate. The aortic perfusion pressure was adjusted to 70-80 mmHg using an adjustable perfusate bypass valve and a bolus of sodium cyanide (50 µl 0.1 % solution) was injected into the perfusate line to transiently stimulate peripheral chemoreceptors (Dutschmann et al., 2000). After partial pulmonectomy, the phrenic nerve was exposed and aspirated into a glass capillary suction electrode pulled to a diameter that ensured adequate seal on the nerve.

The signal was amplified (×10,000; DAM50, WPI) and filtered (band-pass 300 Hz – 1 kHz). Using a computerized data acquisition system (Powerlab, ADInstruments), data were digitized at 1 k Samples/s and digitally integrated through full wave rectification and 50 ms moving average on a duplicate channel. Phrenic burst frequency (f, bursts/min) was determined by counting the number of phrenic bursts occurring during 60 s of normocapnia immediately preceding the gas challenge, the final 60 s of hypercapnia, and 60 s during

normocapnic recovery 5 min after a return to baseline. Phrenic burst amplitude was derived from the mean integrated peak height of all bursts within these 60 s periods, expressed in arbitrary units within each preparation and as a proportional change within a preparation in response to the gas challenge. Neural minute ventilation (NVE) was calculated as the product of burst frequency and amplitude, again expressed in arbitrary units and as a proportional change with treatment (Eldridge, 1971).

Gas Challenges: Preparations were maintained on normocapnic perfusate for at least 1 h. For the gas challenge, gas equilibrating the perfusate was switched in sequence to 5-min periods of hypocapnia (96.5 % O_2-3.5 % CO_2; pH 7.5; P_{CO_2} ≈ 23 mmHg) followed by 5-min periods of hypercapnia (91 % O_2-9 % CO_2; pH 7.2; P_{CO_2} ≈ 60 mmHg) and subsequently returned to baseline normocapnia. The hypercapnic challenge conditions approximated those occurring during a 4 % increase in inspired CO_2.

Pharmacological challenges: Nc- and IHc-pretreated preparations were tested with the addition of an antagonist to disrupt particular neurotransmitter signaling mechanisms. Pharmacological agents were added after the 60-min normocapnic period, which was maintained for an additional 10 min prior to gas challenge. Ketanserin tartrate (5 μM, Sigma) was administered to induce CO_2 chemoresponsive dysfunction and determine the influence of removing ketanserin-sensitive (presumably 5-HT_2 receptor-mediated; Corcoran et al., 2013) processes on responsiveness to the gas challenge. We have previously shown that ketanserin-sensitive mechanisms are critical for chemoresponsiveness in this system (Corcoran et al., 2013; Mosher et al., in review). IHc- and Nc-pretreated animals

at each of the three developmental ages were tested for ketanserin-insensitive CO_2 chemoresponsiveness. Comparing the effects of these treatments demonstrated the CO_2 chemoresponsiveness of this preparation and the dependence of the IHc-induced response on ketanserin-sensitive and insensitive mechanisms.

Data and statistical analyses: Ventilatory parameters were quantified from the recorded neurograms. Frequency (f; the number of bursts per unit time), amplitude (the mean peak voltage of bursts), and a neural correlate of minute ventilation (NVE; frequency•amplitude) were calculated from the bursts occurring in the last minute of a gas challenge (normocapnia or hypercapnia). As the ventilatory response to hypercapnia may not be adequately reflected by only a change in f, we also chose to report amplitude and NVE. CO_2 responsiveness was quantified as a hypercapnic response percentage calculated by expressing the hypercapnic frequency, amplitude or NVE as a percentage of that parameter's first normocapnia value, which was recorded at the end of the stabilization period.

Data Parsing: We and others have demonstrated that the *in situ* rat brainstem preparation exhibits clear responsiveness to hypercapnia and provides an appropriate model for the study of mechanisms contributing to CO_2 chemosensitivity (Corcoran et al., 2013; Day and Wilson, 2007; Iceman et al., 2013; Mosher et al., in review; Toppin et al., 2007). These prior studies have illustrated that the response is most clearly manifest in changes in burst frequency and the index of minute ventilation, NVE. Our pilot investigations suggested the possibility that preparations exhibiting a relatively high initial burst

frequency have a lower apparent responsiveness to hypercapnia, which suggests a frequency limitation. The present study was designed to determine CO_2 chemosensory mechanisms, necessitating a focus of responsive preparations free of potential confounding factors.

To remove the potential confounding influence of dampened CO_2 chemoresponsiveness in preparations with elevated baseline burst frequencies, we subjected the data to the following parsing protocol. In preparations exposed to neither pharmacological manipulation nor IHc, we plotted the relationship between hypercapnic responsiveness and initial burst frequency and determined a linear regression ($P < 0.001$; $R^2 = 0.74$) described by the equation: hypercapnic responsiveness (HcR) = 192 – 3.45 (initial frequency). The highest sensitivity observed in any of these preparations was 184.6. A half-maximum value was used to determine a threshold initial burst frequency identifying preparations for which high initial frequency could confound subsequent CO_2 responsiveness. Preparations having an initial burst frequency above this threshold were not included in the dataset. When so parsed, 3 of 49 preparations (6 %) were removed from analyses; a total of 46 preparations contributed to the data set.

One-way repeated-measures analysis of variance (RM-ANOVA) was used to compare f, amplitude or NVE before and during hypercapnia ketanserin treatment, which quantified hypercapnic responsiveness for these three ventilatory parameters under ketanserin treatment. One-way ANOVA was used to compare the effect of drug treatment on the hypercapnia-induced change in each parameter. For each parameter (f, amplitude

and NVE) measured on each animal in each experiment we calculated the % change from normocapnia = [(parameter during hypercapnia – parameter during normocapnia) / parameter during normocapnia)] • 100%. Values reported in the text are mean ± standard error. To compare the mean hypercapnic responsiveness for each parameter between groups, a Tukey post-hoc analysis was used following a significant ANOVA.

4.4 Results

Burst discharge recordings: Phrenic neurogram recordings from all preparations were characteristic of the eupneic pattern typical of this system (St.-John and Paton, 2000). Preparations derived from Nc-pretreated pups before pharmacological manipulation had a mean normocapnic burst frequency of 25.2 ± 2.67 bursts/min.

Hypercapnic responsiveness: Hypercapnic responsiveness was characterized by deriving the proportional change from normocapnic values, expressed as a % change (Figs. 4.2, 4.3, 4.4). In preparations derived from Nc-pretreated pups assessed for their CO_2 chemoresponsiveness at P21-35, ketanserin abolished the normalized hypercapnic burst frequency, NVE and amplitude and responses (Figs. 4.2, 4.3, 4.4). However, in preparations derived from IHc-pretreated pups that also received ketanserin, when assessed for CO_2 chemoresponsiveness at P21-35, burst frequency increased 35 ± 6.4 % with hypercapnia ($P < 0.001$; Fig. 4.2), and NVE also increased 24 ± 4.7 % ($P < 0.001$; Fig. 4.3). In preparations derived from Nc-pretreated pups assessed for their CO_2 chemoresponsiveness at P36-50, ketanserin abolished the normalized hypercapnic burst frequency, NVE

and amplitude and responses (Figs. 4.2, 4.3, 4.4). In preparations derived from IHc-pretreated pups that received ketanserin when assessed for CO_2 chemoresponsiveness at P36-50, both burst frequency and NVE were elevated by hypercapnia, 30 ± 6.6 % (P < 0.001; Fig. 4.2) and 42 ± 15 % (P < 0.001; Fig. 4.3) respectively. In preparations derived from Nc-pretreated pups assessed for their CO_2 chemoresponsiveness at P51-65, ketanserin abolished the normalized hypercapnic burst frequency, NVE and amplitude and responses (Figs. 4.2, 4.3, 4.4). In preparations derived from IHc-pretreated pups that received ketanserin when assessed for CO_2 chemoresponsiveness at P51-65, both burst frequency and NVE were elevated by hypercapnia, 11 ± 8 % (P < 0.001; Fig. 4.2) and NVE by 20 ± 4.6 % (P < 0.05; Fig. 4.3).

4.5 Discussion

Our study assessed pretreatment with intermittent hypercapnia during postnatal development as a modulator of the neuroventilatory response to CO_2. We tested the hypothesis that IHc-pretreatment would sufficiently enhance CO_2 chemoresponsiveness to overcome dysfunction pharmacologically induced by ketanserin administration (which was previously shown to compromise CO_2 chemoresponsiveness; Corcoran et al., 2013; Mosher et al., in review) and that this enhancement of CO_2 chemoresponsiveness would induce long-lasting respiratory plasticity persisting far into life.

CO_2 chemoresponse dysfunction was induced using ketanserin. We have previously shown that disruption of ketanserin-sensitive mechanisms abolishes CO_2

chemoresponsiveness in the rat *in situ* brainstem preparation (Corcoran et al., 2013; Mosher et al., in review). In the present study, ketanserin was again used to induce a pharmacological dysfunction in the signaling mechanisms that appear to be critical for CO_2 chemoresponsiveness. Our results confirm that the rat *in situ* perfused brainstem preparation does exhibit CO_2 chemoresponsiveness (Corcoran et al., 2013; Day and Wilson, 2007; Mosher et al., in review). We also confirmed previous findings that ketanserin-sensitive mechanisms are critical to CO_2 chemoresponsiveness (Corcoran et al., 2013), as ketanserin treatment alone abolishes CO_2 responsiveness in preparations derived from Nc-pretreated animals. The primary influence of ketanserin, in respiratory control mechanisms, is as a post-synaptic 5-HT_2 receptor antagonist (Awouters, 1985, Corcoran et al., 2013). Previous studies investigating CO_2 chemoresponsiveness using the *in situ* preparation have used ketanserin to determine the role of post-synaptic 5-HT_2 receptors (Corcoran et al., 2013). As ketanserin not only blocks 5-HT_2 receptors but also alpha-1 adrenergic (Hoyer et al., 1987) and histamine H1 receptors (Borroto-Escuela et al., 2014), as well as 5-HT_7 and dopamine D1 and D2 receptors with limited affinity (Shen et al., 1993; Wouters et al., 1985), Corcoran et al. (2013) acknowledge that the disrupted response to the hypercapnic challenge may have resulted from a dependence of chemoresponsiveness on alpha-1 adrenergic, histamine H1 or 5-HT_7 receptor activation. However, the primary outcome of ketanserin administration was similar to that resulting from the administration of the 5-HT_{1A} receptor agonist (R)-(+)-8-hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT; Corcoran et al., 2013) which is commonly used in respiratory studies to inhibit 5-

HT neuron transmitter release via activation of hyperpolarizing 5-HT_{1A} autoreceptors (St. John and Paton, 2000). In addition, a similar outcome to ketanserin was also observed following application of the mixed 5-$HT_{1,2}$ receptor antagonist methysergide (Harris et al., 2003). Thus, the most likely conclusion is that elimination of the hypercapnic ventilatory response by ketanserin is due to disruption of 5-HT processes, and that ketanserin-sensitive influences illustrate 5-HT contributions to hypercapnic responsiveness. We attribute our observation of ketanserin-sensitive CO_2 chemoresponsiveness to suggest that 5-HT neurotransmission is critical for CO_2 chemoresponsiveness in this experimental system (Corcoran et al., 2013).

Despite the apparent critical nature of 5-HT-mediated mechanisms confirmed by the above findings, central CO_2 chemosensitivity is best characterized as a complex system function that potentially involves multiple neuron types, brainstem sites, and neurotransmitter mechanisms (Feldman et al., 2003; Mitchell et al., 1990; Nattie, 2009; Nattie and Li, 2009; Putnam et al., 2004). The possibility that diverse mechanisms contribute to such a system function allows for plasticity, providing pH homeostatic regulation despite dysfunction in any one mechanism (Feldman et al., 2003).

We aimed to illustrate that plasticity induced through IHc-pretreatment is long-lasting and capable of overcoming the chemosensory impairment associated with ketanserin-induced dysfunction far into life. We propose that the induced chemoresponsive plasticity enhances multiple CO_2 chemosensory mechanisms and persists far into life. We aimed to induce plasticity within the CO_2 chemoresponse system with IHc-pretreatment

and thereby facilitate the recruitment of non-ketanserin-sensitive chemosensory mechanisms; we aimed to invoke CO_2 chemoresponsiveness when ketanserin-sensitive mechanisms were rendered dysfunctional at various time periods throughout development.

We have previously shown that plasticity induced by IHc-pretreatment, responsible for ketanserin-insensitive responsiveness, was produced through bicuculline- and/or saclofen-sensitive $GABA_{A/B}$ receptor-mediated processes not normally critical for chemoresponsiveness (Mosher et al., in review). As such, we predict the long-lasting chemoresponsive plasticity imparted by IHc-pretreatment may be due to the enhancement of bicuculline- and/or saclofen-sensitive mechanisms. Future studies are needed to confirm this prediction.

Just as different hypoxia stimulus protocols vary in their capacity to evoke respiratory plasticity, hypercapnia stimulus protocols also induce respiratory plasticity to varying degrees. A prior attempt to influence hypercapnic responsiveness with a comparable IHc protocol administered between P7 and P14 showed no influence (Steggerda et al., 2009). However, rat pups exposed to the aforementioned IHc protocol did exhibit an enhanced ventilatory response to a subsequent hypoxic challenge. It is likely that the developmental timing and/or duration of daily IHc stimulus may influence resultant plasticity. Our IHc-pretreatment protocol is the first to elicit long-lasting plasticity that enhances the hypercapnic ventilatory response, and this enhancement persists far into life (at least through P65).

Critique of methods: As ketanserin is a receptor antagonist for several neurotransmitter pathways, future work will need to use more specific pharmacological techniques to investigate the mechanisms that are being enhanced with IHc-pretreatment. Additionally, the *in situ* experimental preparation used in the current study was ideal for the first attempt at investigating the IHc-pretreatment. However, it will be important for future investigations using this IHc-pretreatment protocol to use *in vivo* experimental preparations.

4.6 Conclusion

Our findings are consistent with considerations of central CO_2 chemoresponsiveness as a system function involving multiple neuron types and neurotransmitter mechanisms thus having the potential for considerable plasticity. We propose that IHc-pretreatment is capable of enhancing multiple CO_2 chemosensory mechanisms to result in the maintenance of CO_2 chemoresponsiveness despite ketanserin-sensitive dysfunction and that this plasticity is long-lasting. We show that IHc-pretreatment induces long-lasting plasticity such that CO_2 chemoresponsiveness is maintained despite removal of otherwise critical ketanserin-sensitive mechanisms.

Abnormal homeostatic responses to increased levels of CO_2 are associated with numerous dangerous or life-threatening disorders. It may be that IHc-induced plasticity could enhance homeostatic reflex efficacy and potentially reduce vulnerabilities to these conditions. A number of critical factors remain unknown. These include: identification of

exact bicuculline- and/or saclofen-sensitive mechanisms contributing to CO_2 chemosensory plasticity; changes in ketanserin-sensitive mechanisms with IHc-pretreatment; potential developmental sensitivities to IHc-induced plasticity. The clear potential to induce CO_2 chemosensory plasticity, however, provides possible targets for therapeutic intervention to reverse or offset CO_2 chemoresponsive dysfunction.

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4.8 Figures

See below where figures are displayed on full pages.

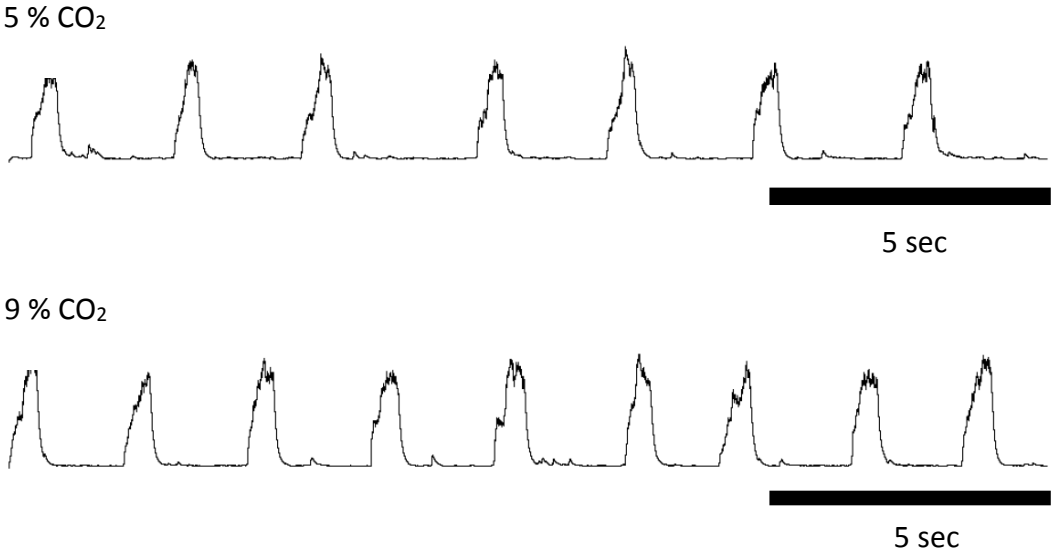

Figure 4.1 **Eupneic phrenic ventilatory burst *in situ.*** Typical phrenic neurogram recording with spikes indicating respiratory bursts. During the *in situ* preparation when the organism was exposed to perfusate containing 9 % CO_2 (hypercapnia; bottom panel) there was an increase in both frequency and NVE (frequency*amplitude) of respiratory events, when compared to experiencing 5 % CO_2 (normocapnia; top panel). Both of the above panels are representative tracings from the last minute of the indicated CO_2 treatment. Tracings taken from a preparation derived from a Nc-pretreated pup without pharmacological manipulation. Tracings reported here are the same used in Chapter 2.

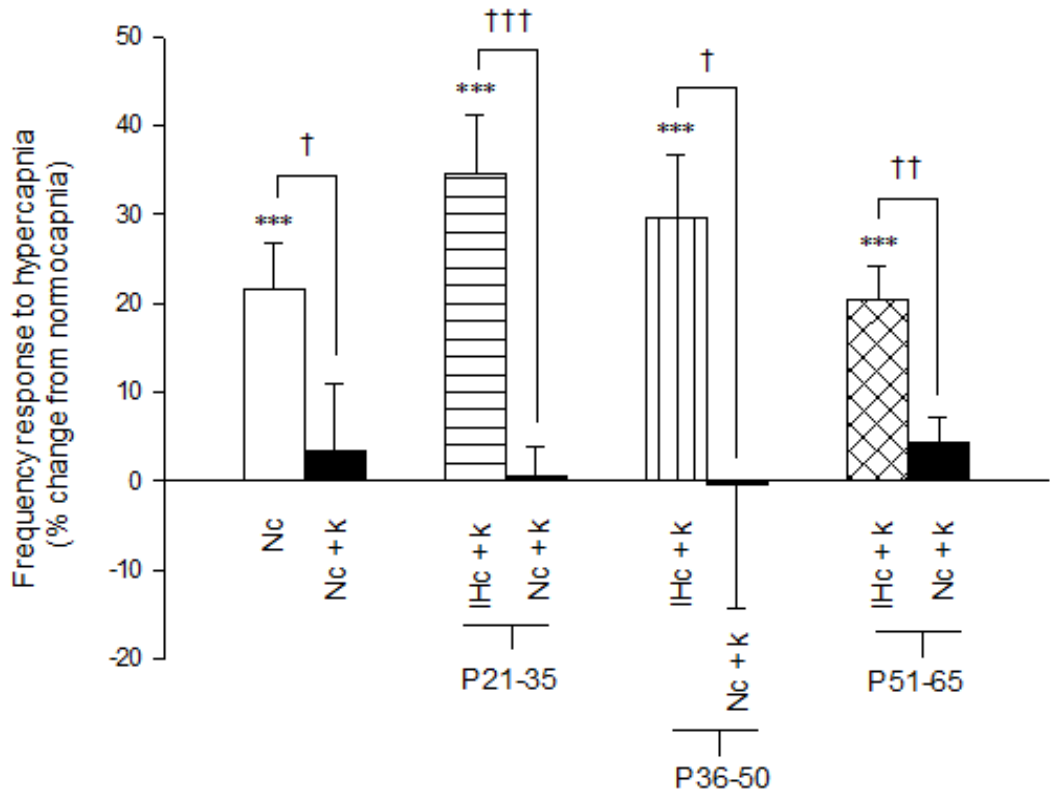

Figure 4.2 **Long-lasting effect of IHc on frequency response to hypercapnia.** The two leftmost bars are previously reported data (Chapter 2) presented here to demonstrate that the frequency response to the hypercapnic challenge is abolished after the administration of ketanserin (k) in preparations derived from animals that were Nc-pretreated (Mosher et al., in review). Hypercapnia failed to increase ventilatory frequency regardless of age of assessment in preparations derived from pups that were Nc-pretreated and received k (P21-35, *n* = 7; P36-50, *n* = 6; P51-65, *n* = 6). Hypercapnia increased ventilatory frequency in preparations derived from IHc-pretreated pups that received k regardless of age of assessment (P21-35, *n* = 10; P36-50, *n* = 8; P51-65, *n* = 9). Each bar represents mean ± SE. Significant increase from normocapnia to hypercapnia (one-way RM-ANOVA): ***P

< 0.001. Between groups comparison of difference in hypercapnic responsiveness determined using one-way ANOVA: †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$.

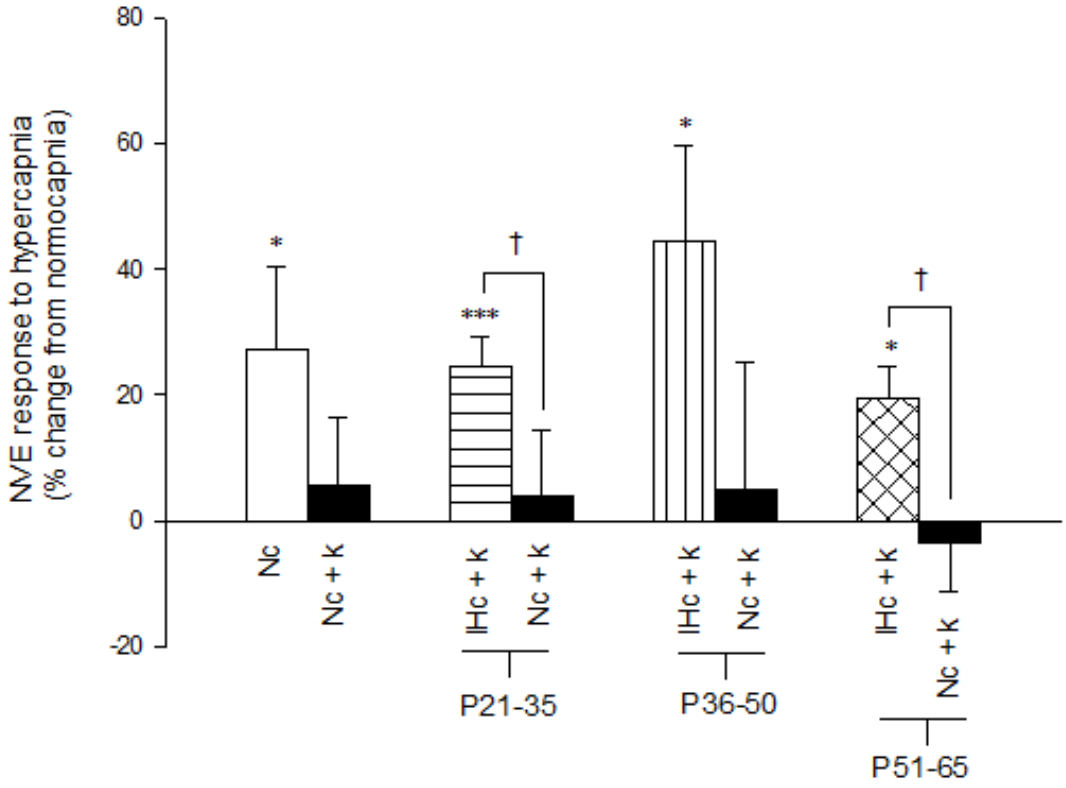

Figure 4.3 **Long-lasting effect of IHc on NVE response to hypercapnia**. The two left-most bars are previously reported data (Chapter 2) presented here to demonstrate that the NVE response to the hypercapnic challenge is abolished after the administration of ketanserin (k) in preparations derived from animals that were Nc-pretreated (Mosher et al., in review). Hypercapnia failed to increase NVE regardless of age of assessment in preparations derived from pups that were Nc-pretreated and received k (P21-35, P36-50 or P51-65). Hypercapnia increased NVE in preparations derived from IHc-pretreated pups that received k regardless of age of assessment (P21-35, P36-50 or P51-65). Each bar represents mean ± SE. Significant increase from normocapnia to hypercapnia (one-way RM-

ANOVA): *$P < 0.05$, ***$P < 0.001$. Between groups comparison of difference in hypercapnic responsiveness determined using one-way ANOVA: †$P < 0.05$.

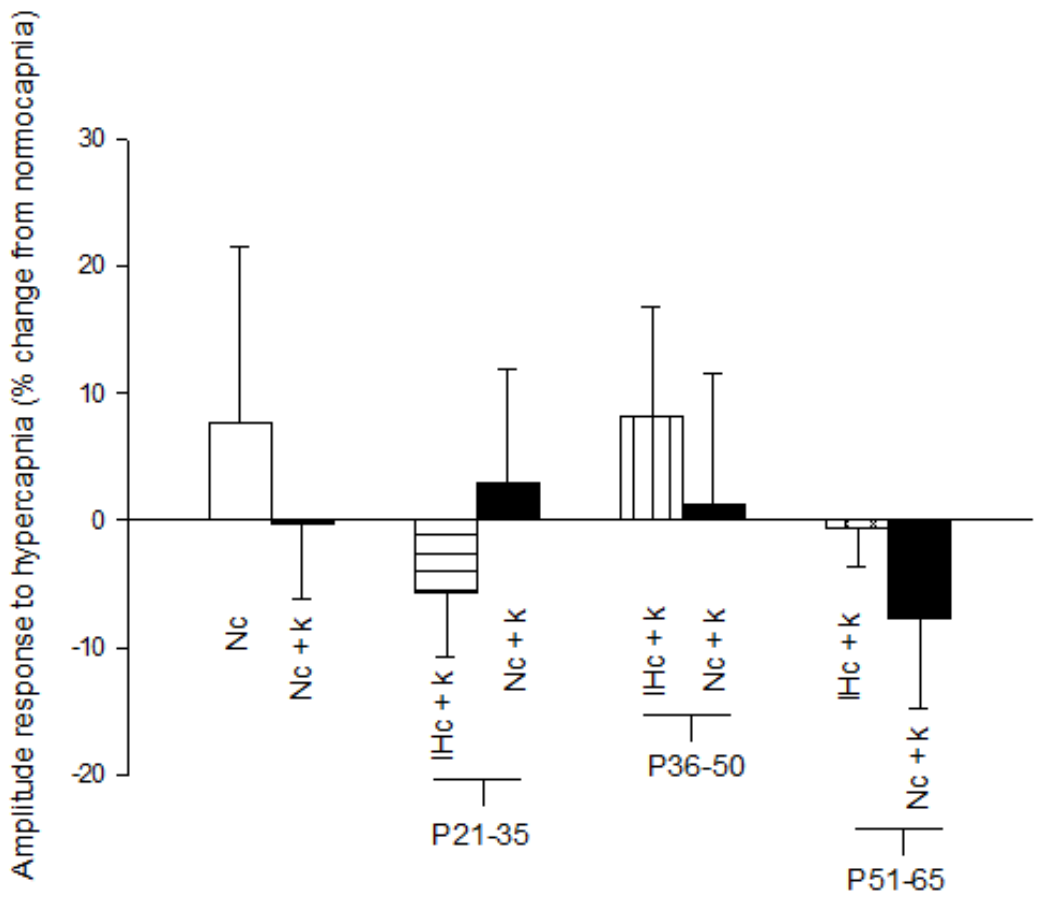

Figure 4.4 **Long-lasting effect of IHc on amplitude response to hypercapnia.** The two leftmost bars are previously reported data (Chapter 2) presented here to demonstrate that hypercapnia failed to increase the ventilatory amplitude response in animals that were Nc-pretreated both with and without ketanserin (k; Mosher et al., in review). Hypercapnia failed to increase ventilatory amplitude for preparations derived from IHc-pretreated pups that received k at any age (P21-35, P36-50 or P51-65). Administration of k also abolished the hypercapnic response for Nc-pretreated animals assessed at all ages P21-35, P36-50 or P51-65). Each bar represents mean ± SE. Between groups comparison

of difference in hypercapnic responsiveness determined using one-way ANOVA failed to reveal any differences between groups.

Chapter 5

Intermittent hypercapnia enhances CO_2 responsiveness and overcomes ketanserin-sensitive dysfunction at various developmental periods[1]

5.1 Abstract

Abnormal homeostatic responses to increased levels of CO_2 are associated with numerous dangerous or life-threatening disorders. We have shown that pretreatment with intermittent hypercapnia (IHc), induces long-lasting respiratory plasticity, due in part to strengthening of bicuculline- and saclofen-sensitive mechanisms. A question remains regarding the existence of a critical developmental period in which IHc-pretreatment is most beneficial. We tested the hypothesis that IHc-pretreatment during postnatal days P12-16 will result in greater increase in CO_2 chemoresponsiveness than other developmental periods and that responsiveness will persist despite ketanserin-induced dysfunction. Rats were exposed to a previously described IHc-pretreatment protocol (Mosher et al., in review) for 5 days beginning at P12, P21 or P36. We subsequently assessed CO_2/pH chemoresponsiveness to a 4 % arterial CO_2 challenge using an unanesthetized juvenile rat *in situ* perfused decerebrate brainstem preparation 10-19 days after IHc-pretreatment. CO_2 chemoresponsive dysfunction was induced pharmacologically with ketanserin. Results indicate that IHc-pretreatment induced CO_2 chemoresponsive plasticity, sufficient

[1] Mosher BP, Taylor BE, Harris MB. Intermittent hypercapnia enhances CO_2 responsiveness and overcomes ketanserin-sensitive dysfunction at various developmental periods. In preparation for publication in *Respir. Physiol. Neurobiol.*

to overcome ketanserin-induced CO_2 chemoresponsive dysfunction, regardless of developmental period. There is no critical period for IHc-induced CO_2 chemoresponsive plasticity.

5.2 Introduction

The development of the respiratory system involves a complex, dynamic network of interconnections that can be influenced by many factors and conditions. Much work has been conducted providing strong evidence for the inherent plasticity of the brain whereby long-lasting or permanent alterations in respiratory control are induced by experience or training during critical periods of development. Although training during a specific developmental period may result in plasticity, such plasticity may not be observed when training is done during a different developmental period, suggesting a critical period. Ample research strongly suggests that such a critical period exists in the respiratory control system of the rat (Liu and Wong-Riley, 2002, 2005; Wong-Riley and Liu, 2005).

In rat at postnatal day 12 (P12), brainstem respiratory nuclei exhibit a distinct, sudden fall in the expression of excitatory neurotransmitters and receptors, and a sharp rise in the expression of inhibitory neurotransmitters and receptors (Liu and Wong-Riley, 2002, 2005; Wong-Riley and Liu, 2005). Evidence suggests that P12 may represent a common sensitive period for most of the brainstem nuclei with known or suspected respiratory control functions (Liu and Wong-Riley, 2005). Because of this transient imbalance

between excitatory and inhibitory neurotransmission, the system may be more vulnerable to respiratory training that may induce plasticity during this time period.

The development of central CO_2 chemosensitivity also appears to develop with a shift in the degree of responsiveness. Wang and Richerson (1999) observed in medullary slices that the percentage of neurons stimulated by hypercapnia was significantly greater in slices from rats older than P12 compared to rats younger than P12. In addition, these findings were also in parallel to those found in medullary raphé neurons in tissue culture (Wang and Richerson, 1999; Wu et al., 2008). These findings may reflect the suggested critical period described by the transient imbalance between excitatory and inhibitory neurotransmission at P12 (Liu and Wong-Riley, 2005).

The purpose of the present study was to investigate the influence of a previously described intermittent hypercapnia protocol (IHc: exposure to mild intermittent hypercapnia; Mosher et al., in review) at different developmental stages. Such IHc-pretreatment has previously been shown to induce long-lasting (through P65) respiratory plasticity by enhancing subsequent CO_2 chemoresponsiveness when IHc-pretreatment is administered during P12-16 (Mosher et al., in review). The present study is an effort to determine if a critical period exists in which IHc-pretreatment has its greatest influence. Based on the rather drastic respiratory control and chemosensory fluctuations observed at P12, we hypothesized that IHc-pretreatment would have its greatest effect when administered during P12-16.

5.3 Methods

Experimental groups: All experiments were done in accordance with the guidelines of the "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health and were approved by the University of Alaska Fairbanks (UAF) Institutional Animal Care and Use Committee. Seven naïve rat dams received normal rat chow and water *ad libitum*, and they were bred with 7 males. Sprague-Dawley rats were used in all experiments (Simonson Laboratories). Resulting pups also received food and water *ad libitum* and were housed and maintained in the UAF Animal Care Facility on a 12 h light/dark cycle. A total of 36 animals from both sexes were used in these studies.

Gas pretreatments: Rat pups were pretreated with intermittent hypercapnia (IHc; 8 consecutive cycles of 5 min 5 % CO_2:balance air, followed by 10 min air) for 5 consecutive days beginning at P12, P21 or P36. Entire litters were randomly assigned to receive IHc-pretreatment at one of these developmental periods. Because related litter-mates rather than randomly assigned individuals were used as experimental subjects, multiple litters (2 to 3) received IHc-pretreatment at each developmental period. During IHc-pretreatments, dams were separated from pups and the home cage was transported to a procedure room adjacent to the animal holding area. Cages were fitted with an airtight lid with a gas inflow and outflow. In this manner, litters (ranging from 2 to 10 pups) were exposed to isothermic inlet gas flowing at 10 l/min through a countercurrent heat exchanger. The gas outlet was connected to 1 m of 5.5 mm internal diameter tubing to pre-

vent room air infiltration without creating substantial positive pressure within the chamber. For the IHc-pretreatment protocol, a programmable digital timer and solenoid valve automatically switched inlet gases between the two sources. This apparatus produced a 10-min period of normocapnia followed by 8 repeated cycles of 5-min hypercapnia and 10-min normocapnia. Valve cycling was monitored using a computerized data acquisition system (LabChart 7, ADInstruments). Pilot studies indicated that CO_2 levels in the enclosure equilibrated with inlet gas concentrations within 90 s, and chamber gas was not monitored during these treatments. After this exposure protocol, cages were removed from the enclosure, dams were returned to pups in the home cage and cages were returned to the adjacent housing facility. In no cases did pup abandonment occur following these brief maternal separations, and patterns and durations of maternal separation were equal between all treatment groups. Procedures were repeated for the same durations on 5 consecutive days, at approximately the same time each day.

In situ assessment of CO_2 chemoresponsiveness: Ten to 19 days following IHc-pretreatment, hypercapnic ventilatory responsiveness was assessed using the *in situ* arterially perfused brainstem preparation as previously described (Corcoran et al., 2013; Toppin et al., 2007, after Paton, 1996). Briefly, animals were anesthetized with isoflurane (5 %, vaporized in 100 % O_2) and pretreated with heparin sodium (500 units, I.P.). Animals were bisected subdiaphragmatically and submerged in an ice-chilled artificial cerebral spinal fluid (aCSF) containing (mM in H_2O): $MgSO_4 \cdot 7H_2O$, 1; $NaH_2PO_4H_2O$, 1.25; KCl, 4; $NaHCO_3$, 24; NaCl, 115; D-glucose, 10; $CaCl_2 \cdot 2H_2O$, 2). The forebrain rostral to the colliculi

was removed by aspiration, and fur, skin and viscera were removed. The diaphragm was separated from the body wall with care taken to ensure integrity of the phrenic nerve. The preparation was moved to the recording station where the descending aorta was cannulated using a double-lumen catheter (Ø 1.25 mm, Braintree Scientific) and perfused retrogradely from a reservoir containing 350 ml aCSF (with 13 g/l ficoll 70, Sigma, added as an osmotic agent). The perfusate was first equilibrated with 95 % O_2-5 % CO_2 (normocapnia, pH 7.4, P_{CO_2} of 33 mmHg). Equilibration mixtures were produced from O_2 and CO_2 using a precision gas mixer (GSM3, CWE) and verified with a CO_2 analyzer (CD-3A, Applied Electrochemistry). Normocapnic (baseline) conditions approximated normocapnic plasma *in vivo*. Lacking hemoglobin, solution hyperoxia (Po_2 ≈ 600 mmHg) was necessary to maintain O_2 content sufficient to meet tissue metabolic demands. This unavoidable hyperoxia was constant under all conditions. Perfusate was warmed to 32 °C, and circulated through a bubble trap and particle filters (25 µm, 45 µm; Millipore) prior to entering the aorta. Perfusate passing through the animal was collected and recycled to the reservoir. The neuromuscular blocker gallamine triethiodide (20 mg/l), and the vaso-constriction hormone vasopressin (5 µM) were added to the perfusate. The aortic perfu-sion pressure was adjusted to 70-80 mmHg using an adjustable perfusate bypass valve and a bolus of sodium cyanide (50 µl 0.1 % solution) was injected into the perfusate line to transiently stimulate peripheral chemoreceptors (Dutschmann et al., 2000). After par-tial pulmonectomy, the phrenic nerve was exposed and aspirated into a glass capillary suction electrode pulled to a diameter that ensured adequate seal on the nerve.

The signal was amplified (×10,000; DAM50, WPI) and filtered (band-pass 300 Hz – 1 kHz). Using a computerized data acquisition system (Powerlab, ADInstruments), data were digitized at 1 k Samples/s and digitally integrated through full wave rectification and 50 ms moving average on a duplicate channel. Phrenic burst frequency (f, bursts/min) was determined by counting the number of phrenic bursts occurring during 60 s of normocapnia immediately preceding the gas challenge, the final 60 s of hypercapnia, and 60 s during normocapnic recovery 5 min after a return to baseline. Phrenic burst amplitude was derived from the mean integrated peak height of all bursts within these 60-s periods, expressed in arbitrary units within each preparation and as a proportional change within a preparation in response to the gas challenge. Neural minute ventilation (NVE) was calculated as the product of burst frequency and amplitude, again expressed in arbitrary units and as a proportional change with treatment (Eldridge, 1971).

Gas Challenges: Preparations were maintained on normocapnic perfusate for at least 1 h. For the gas challenge, gas equilibrating the perfusate was switched in sequence to 5-min periods of hypocapnia (96.5 % O_2-3.5 % CO_2; pH 7.5; P_{CO_2} ≈ 23 mmHg) followed by 5-min periods of hypercapnia (91 % O_2-9 % CO_2; pH 7.2; P_{CO_2} ≈ 60 mmHg) and subsequently returned to baseline normocapnia. The hypercapnic challenge conditions approximated those occurring during a 4 % increase in inspired CO_2.

Pharmacological challenges: IHc-pretreated preparations were tested with the addition of an antagonist to disrupt particular neurotransmitter signaling mechanisms. Phar-

macological agents were added after the 60-min normocapnic period, which was maintained for an additional 10 min prior to gas challenge. Ketanserin tartrate (5 μM, Sigma) was administered to induce CO_2 chemoresponsive dysfunction and determine the influence of removing ketanserin-sensitive (presumably 5-HT_2 receptor-mediated; Corcoran et al., 2013) processes on CO_2 responsiveness to the gas challenge. We have previously shown that ketanserin-sensitive mechanisms are critical for CO_2 chemoresponsiveness in this system (Corcoran et al., 2013; Mosher et al., in review). IHc-pretreated animals were tested for ketanserin-insensitive CO_2 chemoresponsiveness. Comparing the effects of these treatments demonstrated during which developmental period IHc-pretreatment had its greatest influence on ketanserin-insensitive CO_2 chemoresponsiveness.

Data and statistical analyses: Ventilatory parameters were quantified from the recorded neurograms. Frequency (f; the number of bursts per unit time), amplitude (the mean peak voltage of bursts), and a neural correlate of minute ventilation (NVE; frequency•amplitude) were calculated from the bursts occurring in the last minute of a gas challenge (normocapnia or hypercapnia). As the ventilatory response to hypercapnia may not be adequately reflected by only a change in f, we also chose to report amplitude and NVE. CO_2 responsiveness was quantified as a hypercapnic response percentage calculated by expressing the hypercapnic frequency, amplitude or NVE as a percentage of that parameter's first normocapnia value, which was recorded at the end of the stabilization period.

One-way repeated-measures analysis of variance (RM-ANOVA) was used to compare f, amplitude or NVE before and during hypercapnia for ketanserin treatment, which quantified hypercapnic responsiveness for these three ventilatory parameters under ketanserin-induced dysfunction. One-way ANOVA was used to compare the effect of drug treatment on the hypercapnia-induced change in each parameter. For each parameter (f, amplitude and NVE) in each experiment we calculated the % change from normocapnia = [(parameter during hypercapnia – parameter during normocapnia) / parameter during normocapnia)] • 100%. Values reported in the text are mean ± standard error. To compare the mean hypercapnic responsiveness for each parameter between groups, a Tukey post-hoc analysis was used following a significant ANOVA.

5.4 Results

Burst discharge recordings: All our recorded phrenic neurograms displayed a "eupneic" pattern (Fig. 5.1) characteristic of this preparation (Eldridge, 1971 St.-John and Paton, 2000), and displayed this pattern throughout normocapnia, hypercapnia and recovery from normocapnia and drug treatment.

Preparations derived from pups that received IHc-pretreatment from P12-16 had a mean normocapnic burst frequency of 21.4 ± 2.31 bursts/min. Preparations derived from pups that received IHc-pretreatment from P21-25 exhibited a mean normocapnic burst frequency of 12.3 ± 1.03 bursts/min and preparations derived from pups that received IHc-pretreatment from P36-40 had a mean normocapnic burst frequency of 13.9

± 1.36 bursts/min, both of which were lower than preparations derived from pups that had received IHc-pretreatment from P12-16 ($P < 0.001$ and $P < 0.01$ respectively). Burst amplitude was expressed in arbitrary units that were consistent between groups. The neural correlate of minute ventilation (NVE) was calculated as the product of burst frequency and burst amplitude. Normocapnic NVE was consistent between groups with the exception of preparations derived from pups that received IHc-pretreatment from P12-16 exhibited a higher NVE than preparations from pups that had received IHc-pretreatment from P21-25 ($P < 0.01$).

Response to hypercapnia: All preparations were assessed for ketanserin-insensitive CO_2 chemoresponsiveness 10-19 days after IHc-pretreatment at their respective developmental periods. In preparations derived from pups that received IHc-pretreatment from P12-16, hypercapnic responsiveness of f and NVE were 34.5 ± 6.37 % (Fig. 5.2) and 24.4 ± 4.68 % (Fig. 5.3), respectively. One-way RM-ANOVA indicated that these hypercapnia-induced changes were significant ($P < 0.001$ for f; $P < 0.001$ for NVE). No significant responsiveness was resolved for burst amplitude alone (Fig. 5.4; $P = 0.13$).

In preparations derived from pups that received IHc-pretreatment from P21-25, hypercapnic responsiveness of f and NVE were 25.3 ± 6.50 % (Fig. 5.2) and 27.0 ± 13.2 % (Fig. 5.3), respectively. One-way RM-ANOVA indicated that the hypercapnia-induced change in frequency was significant ($P < 0.001$). No significant responsiveness was resolved for NVE (Fig. 5.3; $P = 0.12$) or burst amplitude alone (Fig. 5.4; $P = 0.50$).

In preparations derived from pups that received IHc-pretreatment from P36-40, hypercapnic responsiveness of f and NVE were 26.9 ± 5.01 % (Fig. 5.2) and 19.7 ± 8.89 % (Fig. 5.3), respectively. One-way RM-ANOVA indicated that the hypercapnia-induced change in frequency was significant ($P < 0.001$). No significant CO_2 responsiveness was resolved for NVE (Fig. 5.3; $P = 0.10$) or burst amplitude alone (Fig. 5.4; $P = 0.06$).

Using a one-way ANOVA, there was no difference in f, NVE or amplitude of ketanserin-insensitive CO_2 chemoresponsiveness between preparations derived from pups that received IHc-pretreatment from P12-16, P21-25 or P36-40.

5.5 Discussion

Our study assessed pretreating with intermittent hypercapnia (IHc) during various postnatal developmental periods as a modulator of CO_2 chemoresponsiveness. The purpose of the present study was to investigate the influence of IHc-pretreatment at different developmental stages. Such IHc-pretreatment has previously been shown to induce long-lasting respiratory plasticity by enhancing subsequent CO_2 chemoresponsiveness when administered during P12-16 (Mosher et al., in review). IHc-pretreatment was administered at various developmental stages in an effort to determine if a critical period exists in which IHc-pretreatment has its greatest influence. Based on the rather drastic respiratory control and CO_2 chemosensory fluctuations observed at P12 (Liu and Wong-Riley, 2005), we hypothesized that IHc-pretreatment would have its greatest effect when administered during P12-16.

Our results confirm that the rat *in situ* perfused brainstem preparation does exhibit CO_2 chemoresponsiveness (Corcoran et al., 2013; Day and Wilson, 2007). CO_2 chemoresponsive dysfunction was induced using ketanserin. We have previously shown that acute disruption of ketanserin-sensitive mechanisms abolishes CO_2 chemoresponsiveness in the rat *in situ* brainstem preparation (Chapter 2; Corcoran et al., 2013; Mosher et al., in review). The primary influence of ketanserin, in respiratory control mechanisms, is as a post-synaptic 5-HT_2 receptor antagonist (Awouters, 1985, Corcoran et al., 2013). Previous studies investigating CO_2 chemoresponsiveness using the *in situ* preparation have used ketanserin to determine the role of post-synaptic 5-HT_2 receptors (Corcoran et al., 2013). As ketanserin not only blocks 5-HT_2 receptors but also alpha-1 adrenergic (Hoyer et al., 1987) and histamine H1 receptors (Borroto-Escuela et al., 2014), as well as 5-HT_7 and dopamine D1 and D2 receptors with limited affinity (Shen et al., 1993; Wouters et al., 1985), Corcoran et al. (2013) acknowledge that the disrupted response to the hypercapnic challenge may have resulted from a dependence of chemoresponsiveness on alpha-1 adrenergic, histamine H1 or 5-HT_7 receptor activation. However, the primary outcome of ketanserin administration was similar to that resulting from the administration of the 5-HT_{1A} receptor agonist (R)-(+)-8-hydroxy-2(di-n-propylamino) tetralin (8-OH-DPAT; Corcoran et al., 2013) which is commonly used in respiratory studies to inhibit 5-HT neuron transmitter release via activation of hyperpolarizing 5-HT_{1A} autoreceptors (St. John and Paton, 2000). In addition, a similar outcome to ketanserin was also observed following application of the mixed 5-$HT_{1,2}$ receptor antagonist methysergide (Harris et al.,

2003). Thus, the most likely conclusion is that elimination of the hypercapnic ventilatory response by ketanserin (Chapter 2) is due to disruption of 5-HT processes, and that ketanserin-sensitive influences illustrate 5-HT contributions to hypercapnic responsiveness. We attribute our observation of ketanserin-sensitive CO_2 chemoresponsiveness to suggest that s5-HT neurotransmission is critical for CO_2 chemosensitivity in this experimental system (Chapter 2; Corcoran et al., 2013). In the present study, ketanserin was again used to induce pharmacological dysfunction in the signaling mechanisms that appear to be critical for CO_2 chemoresponsiveness under normal (Nc-pretreated) circumstances (Chapters 2, 4).

Pretreating with IHc at each of the developmental periods (P12-16, P21-25 and P36-40), greatly augmented subsequent CO_2 chemoresponsiveness despite the disruption of ketanserin-sensitive mechanisms (Figs. 5.2, 5.3). Although IHc-pretreatment resulted in the maintenance of CO_2 chemoresponsiveness, this responsiveness was no different between the groups regardless of developmental period of IHc-pretreatment administration.

We attribute the retention of relatively normal CO_2 chemoresponsiveness, overcoming the expected ketanserin-mediated abolishment of CO_2 chemoresponsiveness, to plasticity imparted by IHc-pretreatment. The organization of respiratory CO_2 chemoresponsiveness as a system function grants the potential for considerable plasticity. Not only could a particular mechanism play a differential role in overall system sensi-

tivity under particular conditions, the involvement of multiple mechanisms may allow homeostatic regulation despite partial dysfunction, injury or disease (Feldman et al., 2003; Mitchell et al., 1990; Nattie, 2009; Nattie and Li, 2009; Putnam et al., 2004). Thus, if one reflex mechanism is dysfunctional, alternative mechanisms may accommodate an appropriate response. Our observations indicate that plasticity initiated with IHc-pretreatment resulted in CO_2 chemoresponsiveness mediated by mechanisms other than the ketanserin-sensitive mechanisms normally critical for CO_2 responsiveness. In addition, the IHc-induced enhancement of CO_2 chemoresponsiveness does not appear to have a critical period. Thus, IHc-pretreatment induces plasticity, sufficient to overcome ketanserin-sensitive dysfunction, regardless of developmental period. It may be that the induced ketanserin-insensitive plasticity is bicuculline- and/or saclofen-sensitive, but future studies are needed to investigate this prediction.

Prior attempts to influence hypercapnic responsiveness showed no influence when a comparable IHc protocol was conducted between P7 and P14 (Steggerda et al., 2009). It is likely that the developmental timing and/or nature of the IHc stimulus influences resultant plasticity.

We show that IHc-induced plasticity, nondiscriminative of the developmental period in which it is administered, is sufficient to overcome profound CO_2 chemoresponsive dysfunctions produced by pharmacological disruption of critical ketanserin-sensitive mechanisms. It may be that IHc-induced plasticity to enhance CO_2 chemoresponsiveness could enhance pH homeostatic reflex efficacy and potentially reduce vulnerabilities to

conditions associated with abnormal CO_2 chemoresponsiveness, such as the Sudden Infant Death Syndrome. A number of critical factors remain unknown. These include: identification of exact mechanisms contributing to enhanced CO_2 chemoresponsiveness plasticity; changes in ketanserin-sensitive mechanisms with IHc-pretreatment; influences of different lengths of IHc protocols. The clear potential to induce chemosensory plasticity, however, provides possible targets for therapeutic intervention to reverse or offset CO_2 chemosensory dysfunction.

Critique of methods: It is important to note that although IHc-pretreatments were administered at the various developmental periods (P12-16, P21-25 or P36-40), Nc-pretreatments were not administered to animals at these developmental periods. Although we predict that Nc-pretreatment at these developmental periods will not alter CO_2 chemoresponsiveness, future investigations will need to confirm these predictions.

5.6 Conclusions

The respiratory control system is capable of exhibiting plasticity induced by experience or training during periods of development. The same experience occurring outside of a critical period may have little or no lasting effect, indicating that the plasticity depends on time windows during ontogeny when development can be altered in response to the external environment. Such a critical period does not exist for our IHc-preconditioning protocol under the experimental conditions that were used.

5.7 References

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5.8 Figures

See below where figures are displayed on full pages.

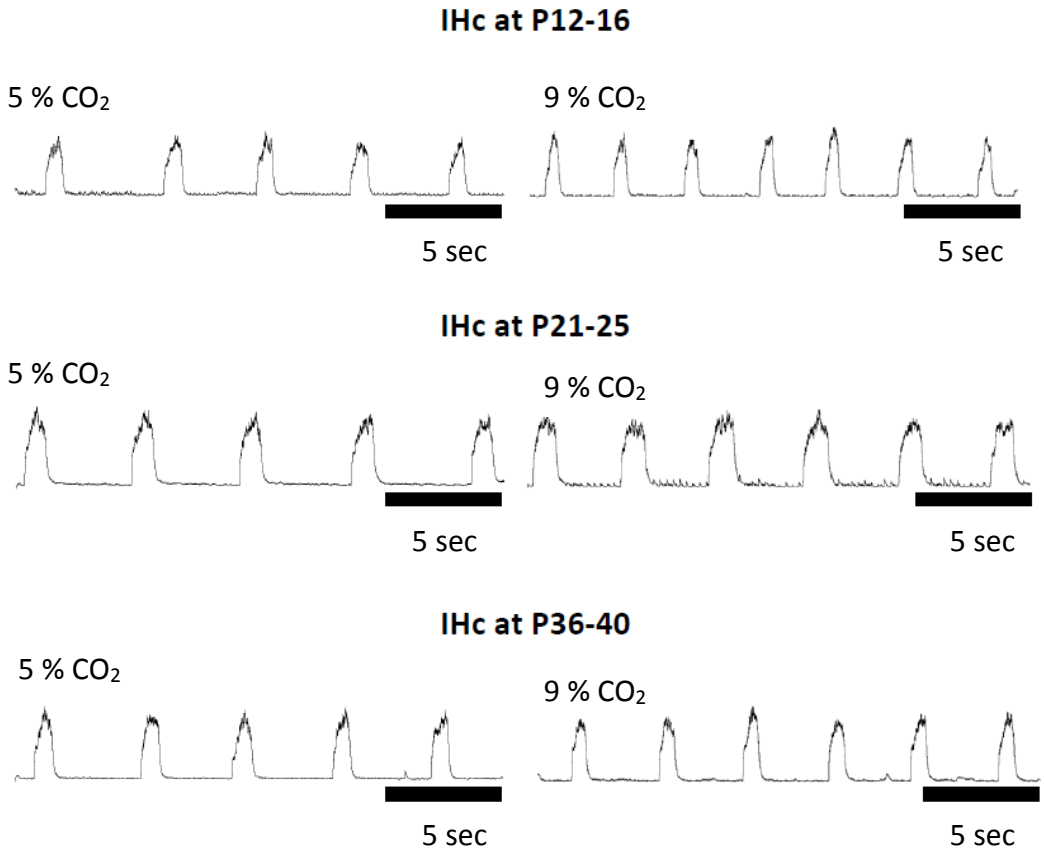

Figure 5.1 **Eupneic phrenic ventilatory burst *in situ* for preparations derived from animals that were pretreated with IHc at various developmental periods.** Typical phrenic neurogram recordings with spikes indicating ventilatory bursts. When the *in situ* preparations were exposed to perfusate containing 9 % CO_2 (hypercapnia; right panels) there was an increase in both frequency and NVE (frequency*amplitude) of ventilatory events compared to 5 % CO_2 exposure (normocapnia; left panels) for animals IHc-pre-treated at P12-16 and P36-40. Preparations derived from pups that received IHc-pretreat-ment at P21-25 exhibited an increase in frequency when exposed to hypercapnia, but no increase in NVE was observed. All panels are representative records of the last minute of the indicated CO_2 treatment.

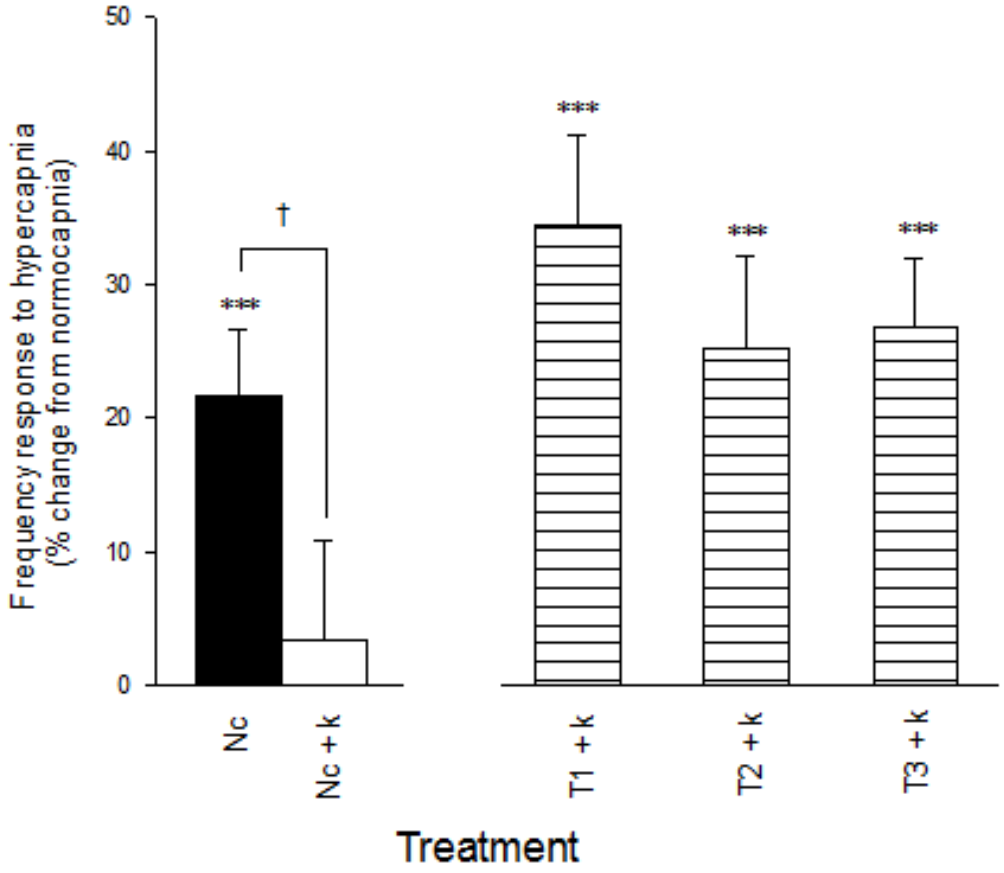

Figure 5.2 **IHc enhances frequency response of hypercapnia regardless of developmental period in which it is administered.** T1 = IHc-pretreatment from P12-16, T2 = IHc-pretreatment from P21-25, T3 = IHc-pretreatment from P36-40. Hypercapnia increased ventilatory frequency, normalized to normocapnia, in preparations, derived from pups that received IHc-pretreatment at T1, and also received ketanserin (k) 10-19 days later during the *in situ* preparation (*n* = 10). Hypercapnia also increased ventilatory frequency in preparations derived from pups that received IHc-pretreatment at T2, and also received k 10-19 days later (*n* = 11). Hypercapnia also increased ventilatory frequency in preparations derived from pups that received IHc-pretreatment at T3, and also received k 10-19

days later (*n* = 15). Bars on left depict data previously described (Chapter 2, Mosher et al., in review) indicating that preparations that received Nc-pretreatment (normocapnic exposures) from P12-16 exhibit a significant response to hypercapnia. However, when preparations derived from Nc-pretreated animals that also received k (Nc + k) the hypercapnic response is significantly reduced. Thus, under control conditions (Nc-pretreated), k abolishes the hypercapnic response (Nc + k bar). However, in preparations from animals that are pretreated with IHc, regardless of developmental period (T1, T2 or T3), that also receive k, the hypercapnic response persists. Each bar represents mean ± SE. Significant increase from normocapnia to hypercapnia (one-way RM-ANOVA): ***$P < 0.001$. Using one-way ANOVA, no difference was found between hypercapnic responsiveness of the groups (T1, T2, T3).

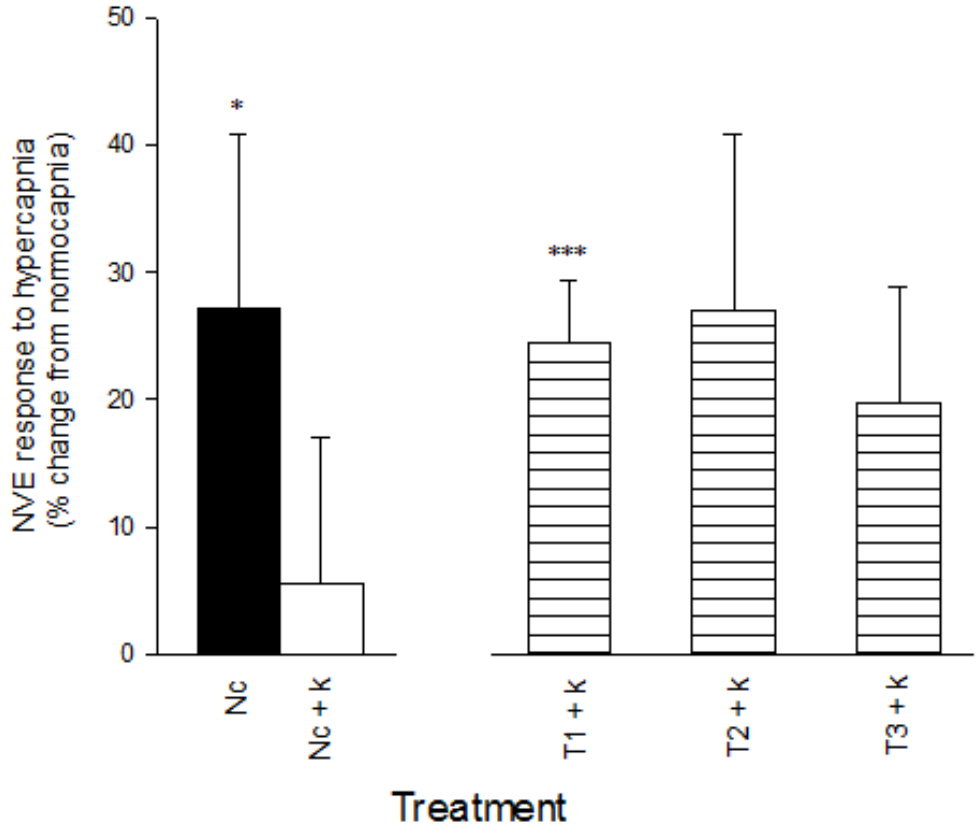

Figure 5.3 **IHc enhances NVE response of hypercapnia when administered at T1**. T1 = IHc-pretreatment from P12-16, T2 = IHc-pretreatment from P21-25, T3 = IHc-pretreatment from P36-40. Hypercapnia increased ventilatory NVE, normalized to normocapnia, in preparations derived from pups that received IHc-pretreatment at T1 and received ketanserin (k) 10-19 days later during the *in situ* preparation (*n* = 10). Bars on left depict data previously described (Chapter 2, Mosher et al., in review) indicating that preparations that received Nc-pretreatment (normocapnic exposures) exhibit a significant response to hypercapnia. However, when Nc-pretreated animals also received k (Nc + k) the hypercapnic response is significantly reduced. Thus, under control conditions

(Nc-pretreated), k abolishes the hypercapnic response (Nc + k bar). Each bar represents mean ± SE. Significant increase from normocapnia to hypercapnia (one-way repeated-measures ANOVA): *$P < 0.05$; ***$P < 0.001$. Using one-way ANOVA, no difference was found between hypercapnic responsiveness of the groups.

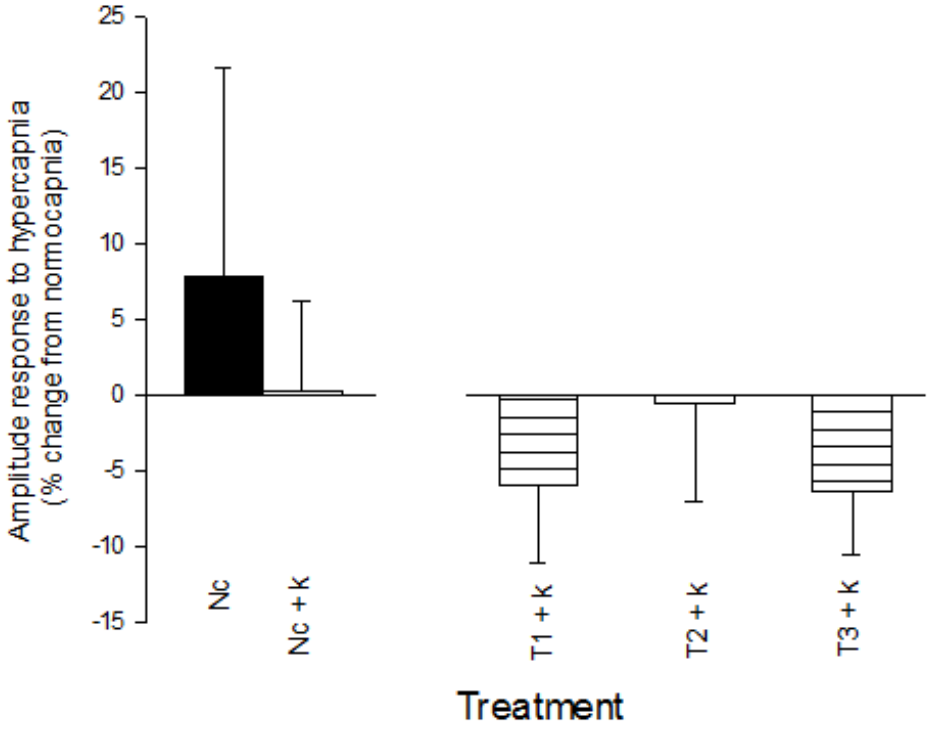

Figure 5.4 **IHc fails to enhance amplitude response of hypercapnia regardless of developmental period in which it is administered.** T1 = IHc-pretreatment from P12-16, T2 = IHc-pretreatment from P21-25, T3 = IHc-pretreatment from P36-40. Hypercapnia failed to increase ventilatory amplitude, normalized to normocapnia, regardless of the developmental period in which IHc-pretreatment was administered when k was also administered. Each bar represents mean ± SE. Using one-way ANOVA, no difference was found between hypercapnic responsiveness of the groups.

Chapter 6

Conclusions

6.1 Summary of results

My results demonstrate the impressive ability of our intermittent hypercapnia (IHc) protocol to induce respiratory plasticity. This is the first time an IHc protocol has been shown to enhance the subsequent response to a hypercapnic challenge. The plasticity induced by IHc-pretreatment is capable of overcoming ketanserin-sensitive dysfunction normally associated with abolishment of CO_2 chemoresponsiveness (Chapter 2; Corcoran et al. 2013; Mosher et al., in review). The plasticity that is induced by the IHc protocol is also capable of overcoming a partial, chronic dietary tryptophan (Trp) restriction that normally results in a measurable reduction of CO_2 chemoresponsiveness (Chapter 3; Penatti et al., 2011). We, and others (Bavis, 2011; Penatti et al., 2011), believe this Trp restriction model be biologically relevant. I predict that, based on previous findings by Penatti et al. (2011), this Trp restriction resulted in a partial reduction in brainstem serotonin (5-HT). Using pharmacological methods I show that IHc-pretreatment acts to enhance bicuculline- and/or saclofen-sensitive (potentially GABAergic) contributions to central CO_2 chemoresponsiveness (Chapter 2). This enhancement of CO_2 responsiveness is so robust that despite two different severities of CO_2 chemoresponsive dysfunction, an appropriate response to a hypercapnic challenge is retained when animals were pretreated with IHc. In addition, the induced plasticity is long-lasting such that animals pretreated with the IHc protocol retain an appropriate response to a hypercapnic challenge far into adult life (at

least through P65), even despite disruption of ketanserin-sensitive mechanisms normally critical for the hypercapnic response (Corcoran et al., 2013). Furthermore, as the administration of the IHc-pretreatment at numerous developmental periods (P12-16, P21-25 or P36-40) resulted in the retention of the hypercapnic response despite disruption of ketanserin-sensitive mechanisms, it appears that IHc-pretreatment induces profound respiratory plasticity regardless of the developmental period in which it was administered. Taken together, these findings demonstrate the impressive ability of our IHc-pretreatment protocol to induced respiratory plasticity overcoming various CO_2 chemoresponsive dysfunctions.

Although much work has been done in an effort to reveal the specific neuronal types and mechanisms involved in the central response to hypercapnia, much less research has been devoted to developing potential therapeutics to combat the array of disorders and diseases associated with the inappropriate response to CO_2. The findings described in the manuscripts within the current dissertation present a potential therapy to reverse an abnormal pH homeostatic reflex associated with numerous respiratory diseases. The clear potential to induce respiratory chemoresponsive plasticity provides possible targets for therapeutic intervention to reverse or offset CO_2 chemoresponsive dysfunction.

6.2 Future directions

The findings reported in the current dissertation describe exciting research, but much work remains to be done and a number of critical factors remain unknown. These include, but are not limited to: identification of exact mechanisms contributing to enhanced CO_2 chemoresponsiveness plasticity; changes in ketanserin-sensitive and bicuculline- and/or saclofen-sensitive mechanisms with IHc-pretreatment; and influences of different lengths of IHc-pretreatment protocols.

The *in situ* perfused brainstem preparation was a very useful preparation in which to test the efficacy of our IHc-pretreatment protocol. However, it is important to replicate the described experiments using an *in vivo* preparation. If, presumably, the administered IHc-pretreatment protocol also functions to enhance CO_2 chemoresponsiveness *in vivo*, it would be important to then test IHc-pretreatment efficacy in another animal preparation (e.g. swine). In addition, it would be very informative to employ genetic tools such as transgenic and optogenetic animals as well as various electrophysiological techniques (e.g. patch clamp) to further investigate the influence of IHc-pretreatment. If my findings are replicated across several species and preparations, a similar protocol could potentially be explored for use in human.

Naturally, much research is necessary before potential therapeutics are able to be used in humans. However, and importantly, the low level of CO_2 utilized in our IHc-pre-treatment protocol (5 %) is safe.

6.3 Critique of methods

The ideal experimental design in studies using rodent neonates would be to assign one pup from each litter to an experimental group. However I felt, as did the IACUC, that such a design would have resulted in the unacceptable sacrifice of a very large number of research animals. To minimize costs, both in terms of financial and animal welfare issues, I chose to accept the limitation of using all pups from each litter in the same experimental group.

Rat pups are poikilothermic and must remain in close proximity to either dams or litter mates to avoid hypothermia. As a result, the gas pretreatment protocol could only be administered to entire litters. Since some of the dams were maintained on a tryptophan-deficient diet, it was impossible to mix pups after birth. In addition, such cross fostering methods are notoriously unsuccessful, and generally result in dam infanticide or pup abandonment. These restrictions required that all pups from each litter were treated the same.

To address the possibility of selection bias and reduce the potential complication of pseudoreplication, multiple litters were assigned to each experimental condition and all pups in all litters were used. I was not able to create a balanced experimental design to include all pharmacological manipulations in pups of a given litter because of the great variation in the number of pups per litter. In addition, when investigating whether or not a critical period existed in which the IHc-pretreatment protocol had its greatest influence,

gas exposures were administered at different developmental periods and tested for responsiveness at different latencies. Again, there was no way to balance this design within a litter without introducing new and much more profound confounding influences resulting from altering litter social structure in some cases and not in others.

Although it may have been possible to redesign the purely pharmacological studies, all experimental protocols needed to be comparable with the dietary tryptophan restriction design, and thus all experimental designs were required to be identical. I was not able to use litter as a variable assessed in a true analysis of covariance as too few litters were assigned to some experimental conditions. I made efforts to address this unavoidable lack of independence between sibling pups when performing the statistics by including litter as a random factor while using a mixed-design ANOVA and a nested ANOVA. However, because of the variable number of pups in each litter and also the variable number of litters in each experimental group, I was not able to use these statistical methods.

Despite these necessary, but potentially concerning shortcomings in experimental design, no single litter of pups presented as an outlier. Taking these issues into account, the lack of independence between sibling pups must be considered when interpreting the data. Future studies will need to randomly assign each pup to a different pharmacological group to address such issues of independence between siblings.

6.4 Potential inadvertent IHc exposures during breast feeding protect against SIDS

There is considerable evidence indicating that infants exclusively breast fed at discharge from the hospital have a significantly lower risk of succumbing to SIDS than infants not breast fed, after controlling for potential confounders (Alm et al., 2002; Ford et al., 1993; Steele and Langworth, 1966). In addition, there has been a significant upward trajectory of mothers choosing to breast feed, and to breast feed for a greater number of months since 1992, correlated with a roughly 50 % decline of SIDS cases (Fig. 6.1; McKenna and McDade, 2005). The mechanism of the protective effect of breast feeding is not clear. One suggested possibility is that infants who are breast fed have a lower incidence of infections due to a stronger immune system because of breast milk consumption and this increased strength of immune system results is a form of protection against SIDS (McKenna and McDade, 2005). Although this may be, I suggest an alternative explanation for the protective role of breast feeding with regard to SIDS.

When an infant is breast fed, the face is in very close proximity to the breast, restricting diffusion of atmospheric gases around the infant. Thus, the infant may be rebreathing its own previously expired air (≈ 5 % CO_2; Bolton et al., 1993) and exposing itself to increased levels of CO_2. In addition, the mother will undoubtedly look down at the breast feeding infant and breathe down on the infant periodically, again increasing the level of CO_2 to which the infant is being exposed. It has been suggested an infant rebreathing its own air results in exposure of about 5 % CO_2 (Bolton et al., 1993), which happens to be the same level of CO_2 I utilized in my IHc-pretreatment protocol. Alternatively, when

an infant is bottle fed, the level of CO_2 rebreathing may be less, potentially resulting in a rather constant level of normocapnic conditions. So, the protective effect of breast feeding may be due to the intermittent exposures of hypercapnia. These exposures may strengthen CO_2 chemoresponsiveness such that if an infant encounters a harmful environmental stressor (e.g. being put to sleep in the prone position), it will be able to respond and appropriately maintain pH homeostasis.

To begin to test this hypothesis, it will first be necessary to measure CO_2 levels around the faces of infants that are breast feeding and compare these with infants that are bottle feeding. In addition, appropriate control groups must also be considered. If it does appear that infants who breast feed are exposed to higher levels of CO_2, compared to bottle fed infants, and these breast fed infants are less likely to succumb to SIDS, nurses and physicians may be able to someday instruct caregivers of newborns to advertently breathe on their infants throughout the day to expose them to intermittent exposures of increased levels of CO_2. This simple instruction may enhance CO_2 chemoresponsiveness and protect against diseases and disorders known to influence the important pH homeostatic reflex.

6.5 Relevance of current dissertation

Given the wide range of diseases and disorders associated with abnormal or dysfunctional homeostatic control of CO_2/pH in the blood, the research described in the current dissertation lays groundwork for much research in the future. As SIDS remains the largest killer

of infants under the age of one year (Moon et al., 2007; Willinger et al., 1991), the finding of a treatment or therapy is imperative. The IHc-pretreatment protocol described here may be the first step in the direction of such a therapy. That being said, much research is needed before a similar IHc-pretreatment protocol can be used to potential protect infants against disorders associated with a dysfunctional CO_2 chemoresponsive, like SIDS.

The laboratory of Dr. Michael Harris at the University of Alaska Fairbanks focuses on central CO_2 chemosensitivity. Most of the previous work in the Harris laboratory has focused on determining the cellular and network interactions involved in the response to hypercapnia, with a special interest in the role that the medullary raphé of the mammalian brainstem plays. Previous work done in the Harris laboratory describing 5-HT and GABA neuronal responses to hypercapnia led to the idea for the work described in the current dissertation.

Although the Harris laboratory does not primarily focus on the interaction of nutrition and the central response to hypercapnia, repeating and confirming the findings previously described by Penatti et al. (2011; Chapter 3) has demonstrated a biologically relevant model for SIDS that may be quite helpful in the Harris laboratory in the future. In addition, although historically the Harris laboratory has not necessarily focused on developing treatments for potential clinical use, my findings reported in this dissertation may provide a platform in which future students in the Harris laboratory can base their investigations.

6.6 References

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6.7 Figures

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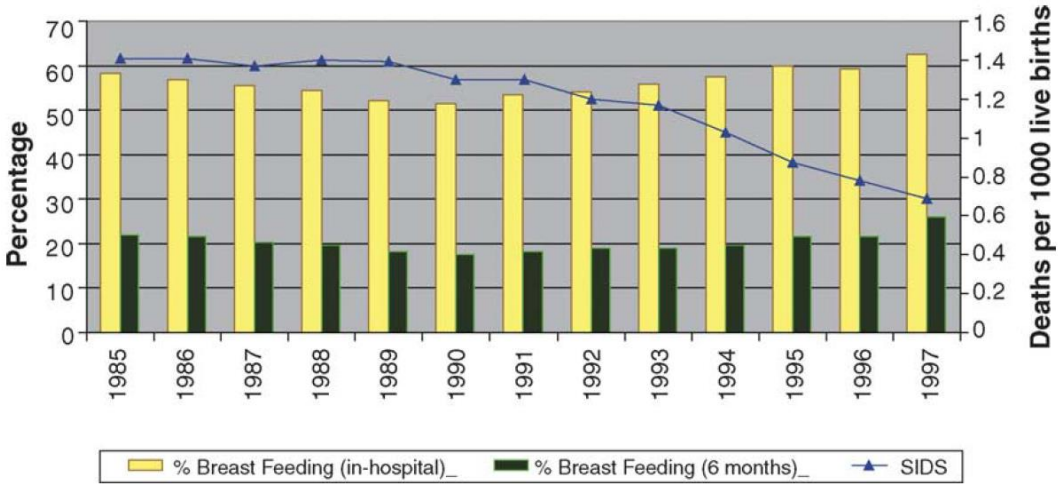

Figure 6.1 **Protective effect of breast feeding with respect to SIDS.** United States SIDS rates and national breast feeding rates (in-hospital and at 6 months) from 1985–1997. The data show that the dramatic decline in SIDS cases, beginning in 1992, occurred in relation to a significant upward trajectory of increased breast feeding. It may be that breast feeding enhances chemoresponsiveness of infants. Modified from McKenna and McDade, 2005.

Appendix

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Institutional Animal Care and Use Committee
909 N Koyukuk Dr. Suite 212, P.O. Box 757270, Fairbanks, Alaska 99775-7270

April 22, 2013

To:	Michael Harris, PhD Principal Investigator
From:	University of Alaska Fairbanks IACUC
Re:	[442123-2] Intermittent hypercapnia and CO2 chemosensitivity

The IACUC reviewed and approved the Amendment/Modification to the Personnel List referenced above by Designated Member Review.

Received:	March 11, 2013
Approval Date:	April 22, 2013
Initial Approval Date:	
Expiration Date:	

This action is included on the May 16, 2013 IACUC Agenda.

The protocol is not approved and the status is modifications required.

PI responsibilities:

- *Acquire and maintain all necessary permits and permissions prior to beginning work on this protocol. Failure to obtain or maintain valid permits is considered a violation of an IACUC protocol and could result in revocation of IACUC approval.*
- *Ensure the protocol is up-to-date and submit modifications to the IACUC when necessary (see form 006 "Significant changes requiring IACUC review" in the IRBNet Forms and Templates)*
- *Inform research personnel that only activities described in the approved IACUC protocol can be performed. Ensure personnel have been appropriately trained to perform their duties.*
- *Be aware of status of other packages in IRBNet; this approval only applies to this package and the documents it contains; it does not imply approval for other revisions or renewals you may have submitted to the IACUC previously.*
- *Ensure animal research personnel are aware of the reporting procedures on the following page.*